

**USE OF NOVEL COMPOUNDS TO REDUCE METHANE PRODUCTION AND
IN PRE-HARVEST STRATEGIES TO DECREASE FOODBORNE
PATHOGENS**

A Dissertation

by

HECTOR GUTIERREZ BAÑUELOS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2008

Major Subject: Animal Science

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ABSTRACT

Use of Novel Compounds to Reduce Methane Production and in Pre-Harvest Strategies
to Decrease Foodborne Pathogens. (May 2008)

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The first aim of this study (Chapter III), the effects of chlorate and nitroethane on foodborne pathogens and rumen fermentation were evaluated. The experimental chlorate product, reduced ($P < 0.001$) fecal, but not ruminal ($P > 0.05$) *E. coli* concentrations by 1000- and 10-fold by 24 and 48 h after chlorate feeding when compared to pre-treatment concentrations ($> 5.7 \log_{10}$ colony forming units/g). Nitroethane treatment decreased ($P < 0.01$) ruminal ($8.46, 7.91$ and $4.74 \pm 0.78 \mu\text{mol/mL h}^{-1}$) and fecal ($3.90, 1.36$ and $1.38 \pm 0.50 \mu\text{mol/g h}^{-1}$) methane-producing activity for treatments 0, 80 and 160 mg nitroethane/kg body weight per day, respectively. Whole animal methane emissions, expressed as L/d or as a proportion of gross energy intake (%GEI) were unaffected by nitroethane treatment ($P > 0.05$).

The second aim of this study (Chapter IV) was conducted to examine the effects of nitroethane and monensin on ruminal fermentation and nitro-metabolizing bacterial populations in vitro. The addition of nitroethane decreased methane production ($\mu\text{mol/mL}$) by at least 90%. The most probable number (MPN) of nitro-metabolizing

bacterial populations was increased ($P < 0.01$) with the addition of nitroethane by at least $3 \log_{10}$ cells/mL compared with monensin, monensin plus nitroethane or the control group.

The final aim of this study (Chapter V) evaluated the effect of two sources of tannins, chestnut (CT) and mimosa (MT) on foodborne pathogens when applied as a hide-intervention and as a feed additive to feedlot cattle. Tannin spray application showed no effect of treatment or application-time ($P > 0.05$) on *E. coli*/total coliforms and total aerobes. Chestnut tannin decreased bacterial load of ruminal *E. coli* and total coliform by at least $0.4 \log_{10}$ CFU/mL. However, fecal *E. coli* concentrations were increased with mimosa by $0.3 \log_{10}$ CFU/g. Also, fecal total coliforms increased with the addition of chestnut or mimosa by at least $0.3 \log_{10}$ CFU/g. Fecal *Campylobacter* concentrations (\log_{10} CFU/g) increased with the addition of chestnut and mimosa by at least $0.4 \log_{10}$ CFU/g.

DEDICATION

With all my love to my mother, Armida Bañuelos Sanchez. My work is a small tribute of her courage, guidance, patient and love. She taught me what I know and set an example of bravery under adverse situations.

Also, I would like to dedicate my work to my Father, Carlos Gutierrez† and my brother, Jorge Carlos Gutierrez, and extend it to Manuel Marin and all my family.

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NOMENCLATURE

ADG	Average Daily Gain
BW	Body Weight
C2	Acetate
C3	Propionate
C4	Butyrate
CH ₄	Methane
CBC	Consecutive Batch Culture
CFU	Colony Forming Units
CO ₂	Carbon Dioxide
DM	Dry Matter
DMI	Dry Matter Intake
FCR	Feed Conversion Ratio
H ₂	Hydrogen
LSD	Least Significant Difference
LSM	Least Square Mean
NE _g	Net Energy for Production
NE _m	Net Energy for Maintenance
MPN	Most Probable Number
NPA	3-Nitro-1-propionic acid
SEM	Standard Error of the Mean

SF ₆	Sulfur Hexafluoride
TDN	Total Digestible Nutrients
VFA	Volatile Fatty Acids

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CHAPTER I

INTRODUCTION

Continued increases in world population growth is raising the demand for animal-protein food products, and creating global environmental challenges. Therefore, under the actual animal production systems, it is desirable to develop strategies such to improve efficiency of feed utilization in the production of high quality meat products in an environmentally sustainable manner. Foodborne pathogens have detrimental effects on the quality and safety of the meat. Consequently, it has been proposed the use of pre-harvest strategies to decrease the risk to acquire a foodborne disease. However, the implementation of those interventions has economical issues that not all the producers are able to acquire. For that reason, in this research, it was tested compounds that besides the possible negative effect on pathogens, also has a probably positive impact improving feed efficiency by decreasing methane production and its environmental costs.

The bovine gastrointestinal tract is a recognized reservoir for important bacterial pathogens such as Shiga toxin-producing *Escherichia coli* O157, *Salmonella* and *Campylobacter*, which have been estimated to cause more than 3.9 million human infections annually (Mead *et al.*, 1999). The economic costs of human infections from those pathogens have been estimated in more than \$4.5 billion each year (USDA/ERS, 2004). The importance of gastrointestinal bovine tracts as pathogens carriers is well known. However, previous research has indicated the importance of hides as a pathogen-

This dissertation follows the style of Journal of Animal Science.

transmitter to beef carcasses, especially for Shiga toxin-producing *E. coli* O157 and non-O157. Therefore, quantitative risk assessments have been indicated that strategies that can reduce concentrations of these bacteria in cattle before they arrive at slaughter plants may significantly reduce human exposures to the pathogens (Hynes and Wachsmuth, 2000; Vugia *et al.*, 2003; LeJeune and Wetzel, 2007).

Ruminants have an important role in providing protein as meat or milk. The digestive tract of ruminants is ideally suited for microbial fermentation. Products of fermentation become available as energy (volatile fatty acids) and protein (microbial source) for the host animal. However, ruminal fermentation also produces methane, carbon dioxide, and ammonia. Because ruminal methane produced is eructated, it is considered a wasteful process representing from 2 to 15% of the gross energy consumed by the animal. Furthermore, methane emissions are recognized as a potent greenhouse gas because the high absorption capacity of infrared radiation and contribution to the ozone layer destruction. The rising concentration of methane is correlated with increasing world populations and currently about 70% of methane production arises from anthropogenic sources and the remainder from natural sources (Moss *et al.*, 2000). For those reasons there is increased worldwide interest in addressing strategies to mitigate methane emissions from domestic ruminant animals.

Because the limited research about alternative strategies that can be used to decrease foodborne pathogens and as strategies to mitigate ruminant methane emissions, we decided to develop the following aims.

The first phase of this study (Chapter III) investigated the effects of 14-d oral nitroethane administration on ruminal and fecal *Escherichia coli* and *Campylobacter*, ruminal and fecal methane-producing and nitroethane-reducing activity, whole animal methane emissions, and ruminal and fecal fermentation balance. Also an experimental chlorate product was fed the day following the last nitroethane administration to determine effects on *E. coli* and *Campylobacter* in Holstein steers.

The second phase of this study (Chapter IV) was focused on the effects of nitroethane and monensin (negative control) on methane production and selected fermentation products using a consecutive batch culture and quantification of nitro-metabolizing bacterial populations using the most probable number determination.

The third phase of this study (Chapter V) examined the effects of two sources of tannins, chestnut and mimosa, on foodborne pathogens when applied as a hide-intervention and as a feed additive to feedlot cattle.

CHAPTER II

REVIEW OF LITERATURE

Ruminal Methane Production

Introduction

Ruminal microbial fermentation generates a variety of products of which most, such as volatile fatty acids (VFA) and microbial protein, are useful to the host animal. However, some fermentation products are recognized as a loss of energy. Ruminal methane (CH₄) production is considered an inefficient digestive process, representing from 2 to 15% of the gross energy consumed by the animal (Johnson and Johnson, 1995; Van Nevel and Demeyer, 1996). That process results in the conversion of potentially energy-yielding substrates into a form that cannot be conserved by the host animal, being released by eructation to the environment. Methane emissions also contribute to global warming (Johnson and Johnson, 1995) by trapping infrared radiation 20 times more effectively than carbon dioxide (CO₂).

Methanogenesis

In the rumen, hydrogen (H₂) is produced during the anaerobic fermentation of carbohydrates. This H₂ can be used during the synthesis of VFA and microbial organic matter. The effects of methane could be reviewed in at least two standpoints. First, in a microbiologic perspective, methane-producing archaea, generally known as methanogens, uses the excess of H₂ from NADH (reduced form of nicotinamide adenine dinucleotide) and CO₂ as the principal substrates to produce CH₄. However two genera

(*Methanosarcina* and *Methanosaeta*) have been described which can convert acetate to CO₂ and CH₄ (Deppenmeier, 2002; Pol and Demeyer, 1988). About 82% of the CH₄ formed comes from H₂ reduction of CO₂, while about 18% is derived from formate. Methane production from acetate is not important because most of this VFA is absorbed by the rumen epithelium (Garcia et al., 2000). The second, in a per se standpoint, it considers CH₄ as a reduced end product of fermentation processes. Methane keeps the partial pressure of H₂ in the rumen contents very low, promoting the regeneration of reduced pyridine nucleotides by H₂ gas formation by hydrogenase activity instead of formation of lactate and ethanol by alcohol- or lactate-dehydrogenases (Van Nevel and Demeyer, 1996). The H₂ partial pressure in methanogenic environments containing an active hydrogen-utilizing community is maintained below 10 Pa. This low pressure let electrons at the redox potential of NADH to be released as H₂. As a consequence, part of the intermediates of the fermentative pathways in syntrophic organisms can be converted to acetyl CoA and finally to acetate, thereby generating ATP via substrate-level phosphorylation (Deppenmeier, 2002). The stoichiometric balance of VFA, CO₂ and CH₄ indicates that acetate and butyrate promote CH₄ production whereas propionate formation conserves H₂ (Wolin, 1960). Therefore, when CH₄ production is decreased propionate production is increased.

Using oligonucleotide probes targeting the small subunit ribosomal RNA (rRNA), Lin *et al.* (1997) estimated that archaea comprised approximately 0.5 to 3.0 of the total rRNA within the rumen microbial community. The last statement agrees with traditional enumeration techniques which estimating archaea community at 2% of the

total rumen microbial population. The most predominant species of methanogens in the rumen are related to the genus *Methanobrevibacter* (Garcia et al., 2000; Wright et al., 2004b), with *M. ruminantium* being the most common in corn-fed cattle (Wright et al., 2007). Other methanogen orders reported include *Methanobacteriales*, *Methanomicrobiales*, and *Methanosarcinales* (Wright et al., 2007).

Strategies to Decrease Methane Production

Decreasing the retention time of feed in the rumen may reduce CH₄ production. Previous research indicated that a 30% decline in CH₄ production was observed when the ruminal passage rate was increased by 50% or more (Okine et al., 1989). When expressed as a proportion of digestible energy, CH₄ losses decreased 1.6% for each unit of increase in feed intake above the maintenance requirement (Johnson and Johnson, 1995).

Numerous strategies for reducing energetic losses associated with ruminal CH₄ production have been investigated. But, the majority of these techniques or products not only inhibited CH₄ production, but also inhibited the beneficial oxidation of H₂ affected by this process (Van Soest and Demeyer, 1996). If H₂ accumulates, hydrogenase activity is unfavourable and alcohol- or lactate-dehydrogenase activity arises. However, if methanogens are present, they maintain low partial pressure of H₂ and reduced nucleotides can be re-oxidized by hydrogenase (Russell and Wallace, 1988).

Alternative approaches that have been used to decrease ruminal CH₄ production include ionophores, defaunation, organic acids, long-chain polyunsaturated fatty acids, anti-protozoa and anti-methanogen vaccines, inhibitors of hydrogenases and carboxylases

involved in methanogenesis, use of condensed tannins, and short chain nitrocompounds (Anderson et al., 2003; McGinn et al., 2004; Min et al., 2003; Moss et al., 2000; Russell and Strobel, 1989; Ungerfeld et al., 2006, 2003; Williams et al., 2007; Wright et al., 2004a).

The actual levels of animal productivity cannot be sustained by forage alone (Nocek and Russell, 1988) pushing scientists to develop strategies for decreasing fermentation losses (e.g., CH₄) or increasing the rate of fermentation acid formation. Additives that modify ruminal fermentation, such as organic acids, yeasts, enzymes and antibiotics, have been used to optimize performance in dairy and beef cattle production (Martin et al., 1999; Russell and Houlihan, 2003). It is known that monensin improved feed efficiency, decreased CH₄ production and had lower acetate to propionate ratio (Thornton and Owens, 1981). However, the use of antibiotics as feed additives in beef cattle and dairy cows has been banned in the E. U. due to risk of antibiotic residues in animal products and the subsequent effects on human health. The risk to develop antibiotic resistant bacteria is the most important concern about the use of ionophore, although a debate has been proposed (Russell and Houlihan, 2003). For this reason, attention has recently shifted to test alternative non-antibiotic products to modify rumen fermentation to decrease CH₄ production.

Natural antimicrobial products have been proposed to increase animal feed efficiency. Saponins are natural detergents found in many plants displaying detergent or surfactant properties. Saponin reported effects included reduction of CH₄ and ruminal ammonia (Santoso et al., 2004), which might be attributed to its ammonia-binding

properties or its inhibitory effects on ciliate protozoa in the rumen (Wallace R. J., 1994; Wang et al., 1998). The β 1-4 Galacto-oligosaccharides (GOS) are produced by transgalactosylation of β -D-galactosidases (lactase) derived from *Bacillus circulans* or *Cryptococcus laurentii*. The GOS have been classified as prebiotics (Gibson and Roberfroid, 1995) and their reported effects on ruminants included decreased CH₄ and increased in propionate production (Santoso et al., 2004). Tannins are polyphenolic plant compounds which have been reported to decrease ruminal gas formation and microbial deamination which also may be due to an interaction of tannins with protein prevented bloat (Min et al., 2003; Min et al., 2006; Min et al., 2005).

Metabolism of Nitrocompounds

The economical importance of poisoning cattle by plants containing 3-nitro-1-propanol (nitropropanol) or 3-nitro-1-propanoic acid (nitropropionic acid) suggested the use of nitroethane. This compound is a less toxic analogue of 3-nitro-1-propanol, the aglycone of miserotoxin. Nitroethane was proposed as an alternative to increase rate of nitro-toxic compound degradation and consequently decrease and eliminate cattle-plant toxicity. Further trials showed that nitroethane and other nitro-compounds may have the potential to be used both as an alternative to decrease CH₄ production in ruminants as well as to decrease foodborne pathogens on feed livestock.

Livestock poisoning by plants, principally *Astragalus* spp, have been reported. According to the toxins and their effects, *Astragalus* species can be classified as species that synthesize aliphatic nitro-compounds (nitropropanol or nitropropionic acid); species causing locoweed poisoning; and species that can accumulate selenium. The

nitropropanol derivatives occur as β -glucosides and the nitropropionic acid compounds can be mono, -di, or -tri glucose esters (J. L. Rios, 1997). Ruminal microbial β -glucosidase and esterase hydrolyze the glucose conjugates to liberate nitropropanol or nitropropionic acid, which could be absorbed in the reticulo-rumen or further metabolized by the rumen microorganisms. If absorbed, nitropropionic acid causes poisoning by irreversibly inactivation of succinate dehydrogenase. Nitropropanol, which is more toxic for cattle, is rapidly and irreversibly converted to nitropropionic acid by hepatic alcohol dehydrogenase (Anderson et al., 2005c). If metabolized, nitrocompounds could be detoxified by a number of rumen bacteria including *Coprococcus*, *Megasphaera elsdenii*, *Ramibacterium*, and *Selenomonas ruminantium* (Majak and Cheng, 1981), also *Bacteroides ruminicola*, *Desulfovibrio desulfuricans*, *Veillonella alcalescens*, and species of *Clostridium*, *Lactobacillus*, and *Peptostreptococcus* have been reported, but the metabolism by these compounds was slight non-specific (Anderson et al., 2005c). It was proposed that the anaerobic degradation occurs via a reductive cleavage at the carbon atom adjacent to the nitro group, with the resultant release of inorganic nitrite, which will be rapidly reduced to ammonia, therefore resulting in its detoxification (Majak et al., 1986).

Because the economical impact of nitropropanol and nitropropionic acid by *Astragalus* intoxication, it was proposed that manipulation of the rumen increasing the numbers of nitrotoxin degrading bacteria could decrease plant toxicity (Majak and Cheng, 1981). Later research found that supplementing cattle with nitroethane, which is a less toxic analog of nitropropanol, increased the rate of nitropropanol metabolism,

demonstrating that rumen environment can be manipulated to enhance microbial detoxification of nitropropanol (Majak et al., 1986). The last trial also showed that nitroethane could be degraded at the rumen level and safely administered to cattle. It was observed the establishment of ruminal bacteria capable of nitropropanol and nitropropionic acid degradation (Anderson et al., 1993), which was later isolated (NPOH1) (Anderson et al., 1996; Anderson et al., 1997) and genetically categorized (*Denitrobacterium detoxificans*) (Anderson et al., 2000c). Besides, it was observed that 3-nitro-1-propionic acid and 3-nitro-1-propanol are reduced by ruminal microbes to β -alanine and 3-aminopropanol, respectively. Also, it was observed that whereas β -alanine was further metabolized by ruminal microbes, aminopropanol was not (Anderson et al., 1993). Growth of *Denitrobacterium detoxificans* was supported by the electron acceptors 3-nitropropanol, 3-nitropropionate, nitrate, 2-nitropropanol, nitroethane, nitroethanol, or 3-nitro-1-propyl- β -D-glucopyranoside (miserotoxin). Formate, lactate, and H₂ served as electrons donors of *D. detoxificans* in the presence of the appropriate nitrocompound (Anderson, 1998; Anderson et al., 1997; Anderson et al., 2000c).

Effects of Nitrocompounds on Rumen Fermentation

It was proposed, after isolation of nitro-metabolizing bacterium *D. detoxificans*, that less toxic nitrocompounds could be used as potent inhibitors of ruminal methanogenesis (Anderson, 1998; Anderson et al., 2003). Although the mechanisms are not well understood, a few nitro-compounds have been effectively tested as antimethanogenic interventions in ruminal contents (Table 2.1). The use of 3-nitro-1-propionate and 3-nitro-1-propanol were proved to negatively affect pure cultures of

Table 2.1. Reported effects of selected nitrocompounds on methane and VFA production in vitro

Nitrocompound	Concentration mM/mL	VFA**				Reference
		CH ₄	C ₂	C ₃	C ₄	
3-nitropropionate	5	-19	NE	+55	+37	(1)
	10	-33	NE	+100	+82	
	20	-69	NE	+250	+85	
Nitrate	5	-15	+51	NE	-22	(1)
	10	-7	+97	NE	-47	
	20	-24	+115	NE	-98	
2-nitropropanol	12	-91	-23	-6	-6	(2)
Nitroethanol	12	-95	-5	+10	+30	
Nitroethane	12	-95	-21	-15	NE	
Nitroethane	2	-58	NE	NE	NE	(2)
	12	-94	NE	NE	NE	
	24	-95	+35	NE	NE	

*Reported effects as a percentage difference with respect respective control groups
NE, No difference $P > 0.05$

Reference: (1) (Anderson, 1998); (2) (Anderson et al., 2003)

**VFA, volatile fatty acids corresponding to acetate, propionate, and butyrate respectively for C₂, C₃, and C₄

Methanobrevibacter ruminantium and *Methanobrevibacter smithii* (Anderson and Rasmussen, 1998). However, the inhibitory mechanism of these two nitroalkanes against methanogenic bacteria needs to be clarified. Earlier studies proposed that nitrate reduction may compete for reduction of H₂ and formate (Allison and Macfarlane, 1988), which are substrates for methanogenesis. Previous experiment using 3-nitro-1-propionate to reduce methane production with or without the presence of nitro-metabolizing bacterium (strain NPOH1) concluded that: 1) inhibition of methane production was independent of the amount of reductant used for 3-nitro-1-propionate reduction; 2) suggests that rather than being used as an alternative electron acceptor, CH₄ production was decreased due to a direct toxic effect of 3-nitropropionate on methanogens (Anderson, 1998). Besides, despite their known potential to be enzymatically reduced by *D. detoxificans* (Anderson et al., 2000c), it was concluded that the nitrocompounds did not serve as an alternative electron sink during in vitro incubations since little if any disappearance of the nitrocompounds was observed (Anderson et al., 2003). Short-chain nitrocompounds have caused some, albeit slight, H₂ accumulations during in vitro ruminal incubations (Anderson et al., 2003), suggesting that microbial interspecies-hydrogen transfer, i.e., the consumption of reducing equivalents generated during fermentation, had not been completely optimized.

Ammonia concentrations decreased with the addition of short-chain nitrocompounds on in vitro incubations of ruminal fluid (Anderson et al., 2003). In contrast, monensin is usually but not always associated with a decreased amount of ammonia accumulations (Garcia-Lopez et al., 1996).

Concentrations of VFA, principally acetate, propionate and butyrate, and the acetate:propionate ratio were not significantly affected with the addition of short-chain nitrocompounds (Table 2.1). Those results suggested that reducing equivalents generated during the fermentations were not necessarily directed toward increased production of these more reduced acids.

Preliminary trials showed that among three nitrocompounds, nitroethane was the most anti-methanogenic compound on three gastrointestinal microbial sources (i.e. chicken ceca, bovine rumen, and ovine rumen). An interesting observation was that on chicken ceca CH_4 was completely depleted, concluding that methanogens harbored in chickens were not as diverse as those in ruminants (Saengkerdsub et al., 2006). Besides, Anderson et al. (2006a), showed that nitroethane had greater CH_4 inhibiting activity than 2-nitropropanol on ewes. Methane-producing activity decreased in 45% and 69% for 24 mg and 72 mg nitroethane/kg BW respectively. However, an apparent adaptation occurs after the fifth day. Also, it was observed that those nitrocompounds did not greatly affect VFA production concluding that fermentative efficiencies associated with microbial interspecies H_2 transfer were not compromised.

Foodborne Pathogens

Introduction

Foodborne pathogens include a variety of bacteria, virus, parasites, and the self induced prions. Many of them colonize the gastrointestinal tract of animals which serves as primary reservoir. Tauxe (2002) estimated the annual burden at 76 million cases of illness, 323,000 hospitalizations per year, and 5,000 deaths, concluding that 1 in 4

Americans get foodborne illness each year, and more than 1 in a 1,000 are hospitalized.

Also, the author suggests that more foodborne pathogens will likely be identified.

Salmonella, *Campylobacter* and *Escherichia coli* O157:H7 have emerged in the last years and represent the majority of foodborne illness.

Increasing antimicrobial resistance is an important trend among foodborne bacterial pathogens and it has been related to the use of antibiotic agents in human populations and also the use of antibiotics in agriculture. The use of a fluoroquinolone in a chicken flock resulted in replacement of prevalent *Campylobacter jejuni* strains from 100% susceptibility to 100% resistance within days. Also, the use of therapeutic third generation cephalosporins in cattle could be associated with the appearance of *Salmonella* strains that are resistant to ceftriaxone (Tauxe, 2002).

Alternative technologies to decrease foodborne pathogens have been evaluated at the gastrointestinal level of the animals. Scientists have proposed that pre-harvest interventions can decrease the risk of foodborne pathogens. Preferred alternatives are those that do not develop antimicrobial resistance.

Prevalence

Campylobacter. It has been recognized as one of the most common causative agent of foodborne pathogens (Vugia et al., 2006), with *C. jejuni* being the most widespread species counting for 99% of all *Campylobacter* infections. The incidence is about 20 cases per 100,000 population diagnosed in the USA (CDC., 2005) although Vugia (2006) reported an incidence of 13 cases per 100,000.

Prevalence in ruminants has been reported at 89% (Stanley and Jones, 2003) with animals generally being asymptomatic. However, it has been known to cause diarrhea and gastroenteritis in some cases. *Campylobacter jejuni* has been detected in the gastrointestinal tract of cattle (Besser et al., 2005), serotypes were isolated from gallbladder, large intestine, small intestine, liver and lymph nodes (Garcia et al., 1985). *Campylobacter* fecal shedding and hide swabs counts were reported higher on feedlot cattle than on pasture cattle (Beach et al., 2002). An apparent transmission of *C. jejuni* among feedlot cattle during the feeding period was also observed, resulting in a high prevalence of *C. jejuni* excretion by cattle approaching slaughter (Besser et al., 2005).

Occurrence of *Campylobacter* on raw products varies depending on geographical zone and post-harvest interventions at specific slaughter plants. However, it has been reported that even though *Campylobacter* was detected in 54% of the cattle, raw meat was not greatly contaminated. Results showed that only 1.3% beef meat samples were positives in comparison with broilers where *Campylobacter* prevalence was 83% and chicken raw meat contaminated at 81% (White et al., 2004). Although poultry has long been considered the principal reservoir for strains resulting in human infection, cattle may also represent an important reservoir host species (Stanley and Jones, 2003).

Escherichia coli. *Escherichia coli* O157:H7 is a leading cause of foodborne illness. It was estimated that 73,000 cases and 61 deaths occur in the US annually (CDC., 2006). Overall incidence per 100,000 populations was 1.06 for Shiga toxin-producing *E. coli* which is the most common serotype of O157:H7, and 0.33 for non-

O157 (Vugia et al., 2006). *Escherichia coli* O157:H7 has been found in the intestines of healthy cattle, goats and sheep.

A good functional rumen is relatively absent of facultative anaerobe such as *E. coli*. Fasting and sporadic feeding perturbed tend to increase the load of *E. coli* and to a less extent *E. coli* O157:H7 (Rasmussen et al., 1999). Previous research concluded that high-grain diets increased the prevalence of *E. coli* O157:H7. Also, the same authors recommended to feed the ruminants with high forage diets before slaughter to decrease the load of coliforms (Diez-Gonzalez et al., 1998). However, other authors dismissed this concept; with the argument that there is a high prevalence of *E. coli* O157:H7 in Argentina, but the cattle is feeding almost exclusively with high forage diets (Rasmussen et al., 1999).

Dietary factors that shown a pattern included: a negative relationship to *E. coli* with cottonseed and clover feeding, and a positive relationship with ionophore feeding. The dietary factors present in cottonseed that could be related are gossypol, which was proved to negatively affect the growth of gram positive bacteria, but little effect was found on *E. coli*. Nevertheless, it is possible that other compounds like terpenoids (in cottonseed) or saponins (in clover) could affect *E. coli*. Feeding forage legumes has also been reported to decrease shedding of *E. coli*, these plants are specially rich in secondary compounds including coumarins. *Escherichia coli* O157:H7 was inhibited by coumarin aglycones, such as aesculetin, umbelliferone, coumarin and scopoletin in vitro (Rasmussen et al., 1999).

Preharvest Interventions

Besides postharvest strategies, methods to reduce pathogens before arrival to the slaughter plants may significantly decrease human illness associated with foodborne pathogens. An achievable objective for preharvest intervention for foodborne pathogens in cattle is to reduce the magnitude and the prevalence of ruminal or fecal excretion. While antimicrobial treatment exists for reducing gut concentrations of bacterial pathogens, their use for preharvest control is not recommended because of antimicrobial pathogen resistance and possible dangerous residues that could be harmful to people. Antimicrobial resistance has been attributed to antimicrobial drug use in animal reservoir populations and subsequent transmission of resistance isolates to human (Smith et al., 1999).

Antimicrobial resistance has been increasing in frequency among *Campylobacter*. Drug resistance was more frequent in *C. coli* than *C. jejuni*, although it was observed that *C. jejuni* is widely distributed and frequently isolated, while *C. coli* is more narrowly distributed (Bae et al., 2007; Bae et al., 2005; Hong et al., 2007). The most common resistance was to tetracycline (82%), erythromycin (54%), nalidixic acid (41%), and ciprofloxacin (35%) (White et al., 2004).

The incidence of *E. coli* was reported higher in ground products compared to whole meats (37% vs 22%). Also it was observed that multiple drug-resistant strains were widespread (85% of isolates). The resistance rates were tetracycline (59%), sulfamethoxazole (45%), streptomycin (44%), cephalotin (38%), ampicillin (35%),

gentamicin (12%), nalidixic acid (8%), chloramphenicol (6%), ceftiofur (4%), and ceftriaxone (1%) (White et al., 2004).

Many preharvest interventions have been proposed to decrease exposure of animal products to foodborne pathogens. Some of them, such as probiotics, prebiotics, and competitive exclusion agents, are based on the concept that beneficial bacteria will fill the same ecological niche of the foodborne pathogens in the gastrointestinal tract, produce a substance or modify the microenvironment of the intestinal tract being inhibitory or deleterious to the pathogen (LeJeune and Wetzel, 2007).

Probiotics are individual or mixtures of bacterial species and yeast, which added in sufficient numbers will provide beneficial health effects to the host (LeJeune and Wetzel, 2007). Competitive exclusion cultures provide protection against pathogens in several ways, including competition for intestinal attachment sites on the mucosa of the intestine, competition for nutrients, and production of compounds which may be toxic to pathogens. Zhao (1998) isolated seventeen strains of *E. coli* and one strain of *Proteus mirabilis* which when administered to cattle prior to exposure to *E. coli* O157:H7 reduced the level of carriage of the pathogen; however, the mechanism by which the carriage was reduced was not elucidated. The probiotics *Lactobacillus plantarum* and *Lactobacillus rhamnosus* inhibited the adherence and attaching and effacing of pathogenic *Escherichia coli* O157:H7 to intestinal epithelial cells. It was proposed that the mechanism was due to an increasing expression of MUC2 and MUC3 intestinal mucins (Mack et al., 1999). The administration of colicin-producing *E. coli* has been shown to displace *E. coli* O157:H7 in cattle, reducing fecal shedding of these pathogens

(Schamberger et al., 2004; Zhao et al., 2003). Colicins are antimicrobial proteins produced under stress conditions by some *E. coli* strains that inhibit other *E. coli* strains (e. g. *E. coli* O157:H7) producing a competitive advantage to the bacteria (Schamberger et al., 2004).

Prebiotics have been used in humans to promote intestinal health. Those are host animal indigestible fructo-oligosaccharides, but are digestible by specific beneficial bacterial species (Doyle and Erickson, 2006). Addition of sorbitol, arabinose, trehalose, and rhamnose displaced *E. coli* O157:H7 from rumen media. However, it was concluded that a good functional rumen with a strong rumen microflora is required to obtain competitive exclusion of the pathogen (de Vaux et al., 2002). Also, it was observed that mannose or commercial non digestible oligosaccharide supplements decreased *E. coli* and *Salmonella*, respectively, in the jejunum of pigs. Moreover, inclusion of lactose in the diets of chickens reduced cecal colonization of *Salmonella* Typhimurium (Doyle and Erickson, 2006).

Other inhibition efforts include the use of phenolic compounds (different kind of tannins), experimental sodium chlorate, vaccination and neomycin. Phlorotannins and berry phenolics have been shown an antibacterial effect, but more in vivo research is necessary. Sodium chlorate is thought to exploit the respiratory nitrate reductase enzymatic pathway. This pathway also co-metabolically reduces chlorate to cytotoxic chlorite ion; bacteria containing nitrate reductase build up toxic levels of this chlorite ion which is lethal to the bacteria.

Preharvest interventions have been proposed to decrease the risk of foodborne pathogens outbreaks (Doyle and Erickson, 2006; Hynes and Wachsmuth, 2000; LeJeune and Wetzel, 2007; Vugia et al., 2003).

Chlorate and Nitroethane

The bactericidal activity of short-chain nitrocompounds has been tested. The oral supplementation of 2-nitro-1-propanol (nitropropanol) to broiler chicks decreased concentrations of *Salmonella enteric* serovar Typhimurium (Jung et al., 2004b). Also, nitropropanol decreased specific growth rates of *S. Typhimurium*, *E. coli* O157:H7 and *Enterococcus faecalis* under in vitro conditions (Jung et al., 2004a). The bactericidal activity of this compound was dependent on concentration and time of exposure. Besides, nitropropanol, 2-nitroethanol and nitroethane showed inhibitory activity against *Listeria monocytogenes* and *L. innocua* in vitro, with nitropropanol being the most effective (Dimitrijevic et al., 2006). Kim et al. (2006) found that nitroethane, nitroethanol, nitropropanol and nitropropionic acid reduced ammonia volatilization in poultry manure by inhibiting growth of uric acid-utilizing microorganisms. The last experiment also showed that nitrocompounds were more successful in inhibiting growth of uric acid-utilizing microorganisms compared to their acid and alcohol counterpart ethanol, propanol, and propionic acid. Recently Horrocks et al. (2007) confirmed the bactericidal activity of nitroethane, nitropropanol, 2-nitroethanol, and 2-nitro-methyl-propionate on *C. jejuni* and *C. coli*; however, the bactericidal effect was pH and concentration dependent. Also it was observed that the nitro-alcohols, nitropropanol and

2-nitroethanol, were more effective than the nitroalkanes, nitroethane and 2-nitro-methyl-propionate, in decreasing the survivability of *C. jejuni*.

Moreover, nitroethane or related nitrocompounds have been shown to significantly enhance (more than 10 folds) the *Salmonella*- and *E. coli*-killing activity of chlorate in swine gut content (Anderson et al., 2006b). However, in this experiment *Campylobacter* was not affected by the addition of nitroethane, whereas earlier research showed that nitrocompounds decreased *Campylobacter* in vitro (Horrocks et al., 2007). The lack of enhance activity in this experiment was partially explained by the dose used which was 10 times lower.

Respiratory nitrate reductase is the terminal component in an electron transport chain accepting electrons from substrate oxidation to reduce nitrate (NO_3^-) to nitrite (NO_2^-) induced by nitrate under anaerobic conditions. In members of *Enterobacteriaceae*, nitrite can be reduced to ammonia. This pathway co-metabolically reduces chlorate to a cytotoxic chlorite ion; bacteria containing nitrate reductase as *E. coli* and *Salmonella* build up toxic levels of this chlorite ion which is lethal to the bacteria (Stewart, 1988). Most members of the family *Enterobacteriaceae* contain the nitrate reductase, whereas beneficial gut bacteria do not. Previous in vitro experiments showed that an experimental chlorate product decreased concentrations of *E. coli* O157:H7 and *Salmonella* Typhimurium DT104 in rumen contents. Also, it was observed that the addition of nontoxic amounts of sodium nitrate to the buffered ruminal fluid increased the bactericidal effect of sodium chlorate on *Salmonella*, suggesting that nitrate was needed to enhance expression of the nitrate reductase activity, but that effect

was not observed on *E. coli* (Anderson et al., 2000a). After those preliminary experiments, it has been proposed that sodium chlorate could possibly be used by the food industry in a preharvest pathogen reduction program (Anderson et al., 2001; Anderson et al., 2005b).

Earlier research showed that sodium chlorate administered intraruminally at 0.2 g per kg of body weight reduced ruminal *E. coli* concentrations. The antimicrobial effect persist beyond the bovine rumen decreasing fecal *E. coli* concentrations, unaffected ruminal fermentation efficiency of the cattle (Anderson et al., 2002). Moreover the addition of 1.2 and 2.3 g of an experimental product containing chlorate per kg of body weight with or without low level of nitrate adaptation decreased fecal *E. coli* concentrations. However, no dose-effect was observed, showing that the dose exceeded that needed to be efficacious, and no enhanced bactericidal activity was observed with the addition of nitrate (Fox et al., 2005). The bactericidal activity over *E. coli* O157:H7 of the experimental chlorate product has been tested in vitro (Anderson et al., 2000a) and in vivo using feedlot cattle (Callaway et al., 2002).

The efficacy of sodium chlorate to reduce enteric pathogens has been tested in many species. In pigs, where drinking water administration effectively decreased *Salmonella* serovar Typhimurium without affect water intake or gut fermentation (Anderson et al., 2004). On chickens, affecting negatively *Salmonella* and preserving most of the microorganism from a competitive exclusion culture (McReynolds et al., 2004). Also, in hens, reducing the incidence and severity of colonization in the crop and ceca and organ invasion by *Salmonella enteritidis* in laying

hens being induced to molt (McReynolds et al., 2005). Besides on sheep decreasing *E. coli* O157:H7 with the addition of an experimental chlorate product with rumen by-pass characteristics preserving fermentation balance (Edrington et al., 2003).

Tannins

Toxicity of tannins towards microorganisms has been studied. Their antimicrobial properties have been shown dependent on concentration, type of tannin, and bacteria strain. However, microbial defense against tannins have been reviewed (Scalbert, 1991).

Chung et al. (1998) tested the antimicrobial properties of tannic acid, propyl gallate and methyl gallate on intestinal bacteria in vitro. It was observed that those tannin compounds decreased strains of *Bacteroides fragilis*, *Clostridium clostridiiforme*, *C. perfringens*, *C. paraputrificum*, *Escherichia coli*, *Enterobacter cloacae*, and *Salmonella* Typhimurium. Besides, the authors concluded that the inhibitory effect of tannic acid could be due to the strong iron binding capacity of tannic acid, whereas the effect of propyl gallate and methyl gallate occurred by a different mechanism. In another experiment, Nagayama et al. (2002) analyzed the bactericidal activity of phlorotannins from brown alga and catechins against foodborne pathogens on mice. In that experiment phlorotannins showed have a stronger bactericidal effect against strains of *Bacillus cereus*, methicillin resistant *Staphylococcus aureus*, *S. aureus*, *Streptococcus pyogenes*, *Campylobacter fetus*, *Campylobacter jejuni*, *Escherichia coli*, *Salmonella enteritidis*, *Salmonella* Typhimurium, and *Vibrio parahaemolyticus* than catechins. Also, it was observed that *Campylobacter* spp were the most susceptible to the phlorotannins.

However the mode of activity was not clarified, but the authors suggested that the interaction of phlorotannins with bacterial proteins could play an important role.

Research on cranberry juice has been arising due to cranberry proanthocyanidins (condensed tannins) effects on P-fimbriated *Escherichia coli* and its relation with human urinary tract infection (Kontiokari et al., 2003). It has been suggested as a mechanistic mode of action an inhibitory effect on the pathogens ability to adhere to uroepithelial cells (Howell and Foxman, 2002). Recently, it was observed that phenolic extract of berries possessed antimicrobial activity against *Helicobacter pylori*, *Bacillus cereus*, *Campylobacter jejuni* and *Candida albicans*. The last two pathogens were more sensible to berries rich in ellagitannins. Also, due to a permeability analysis of examined *Salmonella* strains, it was suggested that the antimicrobial mechanism of berries was due to disintegration of the outer membrane of the bacteria (Nohynek et al., 2006).

Recently, it was suggested that under aerobic conditions the oxidative modification of wattle tannins resulting in hydrogen peroxide production was responsible for *E. coli* sensitivity. Further, an increase in the oxidative stress response, mediated by *oxyR* gene, allowed *E. coli* strains to overcome the inhibitory effect (Smith et al., 2003).

Results from a microbial ecology study showed that condensed tannins switched fecal bacterial populations in the rat gastrointestinal tract, resulting in a shift in the predominant bacteria towards tannin-resistant gram-negative *Enterobacteriaceae* and *Bacteroides* species and returned to pre-exposure population in the absence of dietary tannins (Smith and Mackie, 2004).

Conclusions

The limited alternatives that target methane production with a potential negative impact on foodborne pathogens put forward the objectives of this dissertation. In these series of trials, it was decided to evaluate the use of an experimental chlorate product, nitroethane, and two sources of tannins on methane and foodborne pathogens.

CHAPTER III

NITROETHANE AND CHLORATE EFFECTS ON ENTEROBACTERIA AND GUT FERMENTATIONS*

Introduction

The bovine gastrointestinal tract is a recognized reservoir for enterohemorrhagic *Escherichia coli*, *Salmonella* and, to a lesser extent, *Campylobacter*; bacterial pathogens estimated to cause more than 3.9 million human infections annually (Mead et al., 1999). Human infections by these bacteria are estimated to cost more than \$4.5 billion each year (USDA/ERS, 2004). Quantitative risk assessments indicate that strategies that can reduce concentrations of these bacteria in cattle before they arrive at slaughter plants may significantly reduce human exposures to the pathogens (Hynes and Wachsmuth, 2000; Vugia et al., 2003). Several such strategies are currently being investigated, including vaccination (Potter et al., 2004), the administration of competitive exclusion or colicin-producing *E. coli* (Schamberger et al., 2004; Zhao et al., 1998; Zhao et al., 2003), probiotic *Lactobacillus* spp. (Brashears et al., 2003a; Brashears et al., 2003b), neomycin (Elder et al., 2003) and an experimental chlorate product (Anderson et al., 2002; Anderson et al., 2005a; Callaway et al., 2002; Fox et al., 2005). Additionally, nitroethane has shown inhibitory activity against *Salmonella* and *Listeria* in ruminal contents in vitro

*Reprinted with permission from “Zoonotic bacterial populations, gut fermentation characteristics and methane production in feedlot steers during oral nitroethane treatment and after the feeding of an experimental chlorate product” by Gutierrez-Banuelos, H., R. C. Anderson, G. E. Carstens, L. J. Slay, N. Ramlachan, S. M. Horrocks, T. R. Callaway, T. S. Edrington, and D. J. Nisbet, 2007. *Anaerobe*, 13, 21-31, Copyright [2007] by Elsevier Limited.

(Božić et al., 2005) as well as against *Salmonella* and *Campylobacter* in swine (Anderson et al., 2006b; Jung et al., 2003). Moreover, nitroethane, or related nitrocompounds, have been shown to significantly enhance the *Salmonella*- and *E. coli*-killing activity of chlorate in swine gut contents in vitro and in vivo (Anderson et al., 2006b; Anderson et al., 2007).

While initial results with these aforementioned interventions have been promising, there remains a need to make such strategies economically acceptable for cattle feeders because they likely will be expected to absorb the costs of implementing these interventions. In that regard, the recent work with the nitrocompounds may have application as these compounds are potent inhibitors of ruminal methanogenesis. Methanogenesis, with its concomitant consumption of hydrogen (H_2), plays an important role in maintaining a low partial pressure of H_2 within the ruminal microbial ecosystem which allows fermentation to proceed largely unencumbered by the accumulation of excess reducing equivalents (Miller, 1995). Despite this beneficial role, methane (CH_4) production is recognized as an energetically wasteful process to ruminants, resulting in the loss of 2 – 12% of the gross energy consumed by the animal (Johnson and Johnson, 1995). Methane is also a greenhouse gas that has been implicated in contributing to global warming and ozone layer destruction (Van Soest and Demeyer, 1996). Numerous strategies for reducing energetic losses associated with ruminal CH_4 production have been investigated but the majority of these techniques or products not only inhibited CH_4 production, but also inhibited the beneficial oxidation of H_2 affected by this process (Van Soest and Demeyer, 1996). Changes produced by these inhibitors include

reduction in certain digestive process, microbial growth yields, a decreased production of acetate and an increased production of reduced fermentation acids, notably propionate (Moss et al., 2000). Moreover, inhibition of CH₄ production by these inhibitors often appears to be transient due to the ability of the rumen ecosystem to adapt to ecological perturbations (Van Soest and Demeyer, 1995). In contrast, results so far from two studies have shown that the methane-inhibitor, nitroethane, had little effect on amounts or molar proportions of volatile fatty acids (VFA) produced within in vitro incubations or the ovine rumen thus indicating that this inhibitor may conserve fermentative efficiencies associated with microbial interspecies hydrogen transfer reactions (Anderson et al., 2003; Anderson et al., 2006a).

The objectives of this study were to evaluate the effect of oral nitroethane administration on select fermentation variables and zoonotic bacterial populations in growing steers over a 14-d treatment period both prior to and following a single day feeding (d 15) of an experimental chlorate product.

Materials and Methods

Experimental Design

Eighteen Holstein steers averaging 403 ± 26 (mean \pm SD) kg body weight (BW) were acclimated over a 21-d period a diet containing 50% dry rolled corn, 25% chopped alfalfa, 13% cotton seed hulls, 7% molasses, 3% soybean meal (49% crude protein), and 2% premix (30.26% dry rolled corn, 0.5% COOP Beef TM, 2.5% ADE, 4.56% Vitamin E, 27.33% urea, 14.85 % Limestone, and 20% salt). The NRC predicted nutrient profile (@ 3.0% BW Dry Matter Intake, DMI) was: DM, 89%; TDN, 71%; NEm, 1.65 Mcal/kg;

NEg, 1.03 Mcal/kg; crude protein, 13.3%; calcium, 0.51% and phosphorus, 0.24%.

Steers were randomly allocated ($n = 6/\text{treatment}$) to one of the following treatments: 0, 80 or 160 mg nitroethane/kg BW per day (corresponding to 0X, 1X and 2X treatments, respectively). Steers were penned separately and provided *ad libitum* access to the study diet which was fed in two equal sized meals at 08:00 and 16:30. Feed not consumed was recovered and intake was calculated as the difference between DM offered and refused. Because *Campylobacter* and *Salmonella* prevalence in feedlot steers can be quite variable, all steers were orally inoculated 4 d prior to initiation of treatments with 20 mL of a pooled suspension of freshly collected feces (prepared by combining 10 g feces obtained from each steer with 1 L of phosphate buffer, pH 7.0). Bacteriological cultivation of portions of rumen or fecal samples collected 4 days prior to initiation of treatment revealed that 9 steers were colonized by *Campylobacter* and only one steer was colonized by *Salmonella*. Consequently, in order to provide a better *Salmonella*-challenge, the isolated *Salmonella* was grown overnight at 37°C in Tryptic Soy broth (Becton Dickinson Microbiological Systems, Sparks, MD, USA) and orally inoculated to each steer (9×10^9 CFU/steer) immediately following collection of rumen and fecal samples the day immediately prior to initiation of treatments (day -1).

Upon initiation of treatments, nitroethane was administered as the sodium salt (Majak et al., 1986) twice daily (08:00 and 16:00) by oral gavage. Gavage volumes ranged from 146.2 to 353.1 mL per day depending on dose and individual steer BW. Control steers were administered buffer alone at the same volume basis as steers administered the 2X nitroethane treatment. Ruminal fluid collected by stomach tube and

freshly voided feces were collected approximately 2 h after the morning feeding on days -1, 1, 2, 7 and 14 relative to nitroethane treatment. Specimens were placed immediately into serum vials (ruminal fluid) or whirlpac bags (feces) which were then closed and returned to the laboratory within 1 to 2 h for determinations of volatile fatty acid concentrations, methane-producing and nitroethane-reducing activities and for bacteriological cultivation. Whole animal CH₄ emissions were measured in exhaled gases collected from 09:00 to 07:00 using the sulfur hexafluoride tracer gas technique (Johnson et al., 1994). Pre-evacuated collection canisters were placed on the steers before the morning feeding and were removed after 22 h.

One day after the end of the 14 d nitroethane treatment period, all steers were fed a proprietary experimental chlorate product at 140 mg/kg BW (EKA Chemicals Inc., Marietta, GA, USA) in their last meal and ruminal fluid and feces were again sampled 24 (day 16) and 48 h (day 17) later to determine effects on ruminal and fecal bacteria.

Analytical Procedures

The gas samples were analyzed by gas chromatography to measure CH₄ and sulfur hexafluoride concentrations (Johnson et al., 1994). Volatile fatty acid concentrations were measured by gas chromatography (Hinton et al., 1990) and estimates of CH₄ produced were derived from the fermentation balance of Wolin (1960). Methane-producing activity was determined by in vitro incubation of 5 mL ruminal fluid or 2 g feces, mixed with 5 or 8 mL, respectively, anaerobic dilution solution (Bryant and Burkey, 1953) containing 60 mM sodium formate and 0.2 g finely ground alfalfa (to pass a 4 mm screen). The tubes were capped and incubated 3 h at 39°C under a

hydrogen:carbon dioxide (50:50 mix) atmosphere. At the end of the incubation period, CH₄ concentration was determined by gas chromatography (Allison et al., 1992).

Nitroethane-reducing activity was determined in separate incubations conducted similarly except containing 10 mM added nitroethane; fluid samples collected at 0, 3, 6 and 24 h were analyzed for nitroethane colorimetrically (Majak et al., 1986).

Quantitative cultivation of indigenous *E. coli*, coliforms, *Campylobacter* spp. and *Salmonella* spp. was achieved via plating of serial 10-fold dilutions (in phosphate buffer pH 6.5) to 3M *E. coli*/Coliform Count petrifilm (3M Microbiology Products, St. Paul, MN, USA), Campy Cefex agar (Stern et al., 1992), or Brilliant Green agar (Oxoid LTD, Basingstoke, Hampshire, UK), respectively. Inoculated petrifilm and Brilliant green agar were incubated at 37°C for 24 h. Inoculated Campy Cefex agar were incubated at 37°C 48 h under a microaerophilic gas (10% CO₂, 5% O₂, and 85% N₂). Qualitative cultivation of *Salmonella* was accomplished via overnight enrichment in Tetrathionate broth (Becton Dickinson Microbiology Systems) and further enrichment for 18 to 24 h in Rappaport-Vassiladias R10 broth (Becton Dickinson Microbiology Systems) and selective differentiation on Brilliant Green Agar (Anderson et al., 2000b). Recovered *Salmonella* were serotyped at the National Veterinary Services Laboratory (Ames, IA, USA). Samples were enriched and cultured for *E. coli* O157:H7 using immunomagnetic separation (Elder et al., 2000).

Statistical Analysis

Methane-producing activity, nitroethane-reducing activity, whole animal CH₄ measurements, volatile fatty acid concentrations, ratio of acetate to propionate, estimated

CH₄ production, and log₁₀ transformations of *E. coli* and *Campylobacter* colony forming units (CFU) were analyzed for effects nitroethane treatment, day of treatment, and their interaction using a repeated measures analysis of variance. Means were analyzed using a Least Significant Difference procedure. Due to the magnitude of inter-animal variation in whole animal CH₄ emissions data, an analysis of covariance was conducted using d 0 CH₄ measurements as the covariate for day 7 and 14 measurements. Daily DMI and ADG at the end of the 14 d nitroethane treatment period were analyzed by a completely randomized analysis of variance. Tests for the degree of linear association between measures of whole animal CH₄ emissions, methane-producing activity and estimations of CH₄ production via fermentation balance were accomplished by Pearson correlation coefficients.

Results

Ruminal and Fecal Enterobacteria

Escherichia coli O157:H7 were not recovered from any of the ruminal fecal specimens collected on d -1 of the study, thus none of the subsequent samples were cultured for this bacterium. Moreover, *Salmonella* were recovered only from enriched specimens indicating that concentrations were below our limit of detection (< 10 CFU/g of contents). *Salmonella* were recovered from ruminal and fecal specimens collected on d -1 from 12 of the 18 steers, but from feces of only 1 steer at the end of the 14 d of nitroethane-treatment. Following the experimental chlorate treatment, *Salmonella* were undetectable in ruminal or fecal specimens. For the *Salmonella* isolated pre-nitroethane treatment, 10 were identified as *Salmonella enterica* serovar Heidelberg, which was the

serovar initially isolated and inoculated into all steers on the day immediately preceding initiation of nitroethane administration. The other two isolates were identified as *Salmonella enterica* serovars Anatum and Typhimurium. Two of these isolates (one Heidelberg isolate and the Typhimurium isolate) were recovered from steers allocated to receive the 0X treatment, five of these isolates (all identified as Heidelberg) were recovered from steers allocated to receive the 1X treatment, the remaining 5 isolates (4 identified as Heidelberg and the remaining as Anatum) were recovered from steers to receive the 2X treatment. A single *Salmonella* isolate, identified as serovar Heidelberg, was recovered from feces of 1 steer (from the 2X treatment group) at the end of nitroethane-treatment. Because of the small number of animals in this study and the pattern of qualitative *Salmonella* recovery, these results were not statistically analyzed but rather are presented descriptively only.

Nitroethane treatment had no effect on ruminal or fecal concentrations of generic *E. coli* or *Campylobacter* (Table 3.1). *Campylobacter* spp. were not detected in any of the ruminal fluid samples at any time and were highest ($P < 0.05$) before initiation of nitroethane administration (Table 3.1). An effect of day of treatment was observed on fecal ($P < 0.05$) but not ruminal ($P > 0.05$) concentrations of generic *E. coli*, with concentrations being lower on days 16 and 17 of the study (corresponding to 1 and 2 days post chlorate treatment given on day 15) than before (day -1) or at the end (day 14) of the nitroethane feeding period (Table 3.1). No interaction between chlorate and nitroethane was observed on fecal *E. coli* populations.

Table 3.1. Effect of oral nitroethane administration and chlorate feeding on ruminal and fecal bacterial concentrations in fed steers

Nitroethane treatment (mg nitroethane/kg BW per d)	Generic <i>Escherichia coli</i> (log ₁₀ CFU/g contents)		<i>Campylobacter</i> (log ₁₀ CFU/g contents)	
	Ruminal	Fecal	Ruminal	Fecal
0 (0X)	3.33	4.59	0	2.92
80 (1X)	3.54	4.86	0	3.01
160 (2X)	3.57	4.78	0	1.43
<i>P</i> value	0.7210	0.6536	--	0.2267
SEM	0.23	0.21	--	0.69
Time of treatment (day)				
-1 (1 d pre-nitroethane treatment)	3.26	5.76 ^a	0	1.68 ^b
14 (at end of nitroethane treatment)	3.41	5.92 ^a	0	2.69 ^a
16 (1 d post chlorate treatment)	3.56	2.59 ^c	0	2.65 ^a
17 (2 d post chlorate treatment)	3.70	4.71 ^b	0	2.80 ^a
<i>P</i> value	0.1663	0.0001	--	0.0001
SEM	0.14	0.18	--	0.17
Interaction				
<i>P</i> value	0.9518	0.9555	--	0.9887
SEM	0.25	0.31	--	0.30

^{a, b, c}Means within columns with unlike superscripts differ ($P < 0.05$).

*Ruminal and Fecal Methane-producing and Nitroethane-reducing Activity,
Whole Animal Methane Emissions and Animal Performance*

Oral nitroethane administration decreased ($P < 0.05$) ruminal methane-producing activity, with the activity in steers administered the 2X nitroethane treatment being 40% lower than that observed in steers administered the 0X or 1X nitroethane treatment (Table 3.2). An effect of day of treatment ($P < 0.05$) was observed, with the lowest ruminal methane-producing activity occurring on day 2 and the highest activity occurring on days 7 and 14 of nitroethane administration (Table 3.2). Main effects of nitroethane treatment, day of treatment (Table 3.2) and their interaction on ruminal nitroethane-reducing activity were observed (Figure 3.1).

Fecal methane-producing activity was also decreased ($P < 0.05$) due to nitroethane administration, with main effect means for steers administered the 1X and 2X nitroethane treatment being more than 60% lower than that observed in steers administered 0X nitroethane (Table 3.2). This later finding suggests the passage of effective concentrations of nitroethane to the lower gut. No treatment by day of treatment interaction was observed on either ruminal or fecal methane-producing activity. No effects of nitroethane treatment, day of treatment or their interaction ($P > 0.05$) were observed on fecal nitroethane-reducing activity (Table 3.2).

Whole animal CH_4 emissions (\pm SE), whether expressed as liters produced/d (290.5, 239.1 vs 112.0 ± 38.2 L/d for 0X, 1X and 2X treatments, respectively), or as methane energy as a percentage of gross energy intake (4.31, 3.63 and $4.05 \pm 0.44\%$ GEI

Table 3.2. Effect of oral nitroethane administration on methane-producing and nitroethane-reducing activity in fed steers

Treatment (g nitroethane/kg BW per d)	Methane-producing activity (μmol methane/g contents per h)		Nitroethane-reducing activity (μmol nitroethane/g contents per h)	
	Ruminal	Fecal	Ruminal	Fecal
0 (0X)	8.46 ^a	3.90 ^a	0.05 ^b	0.05
80 (1X)	7.91 ^a	1.36 ^b	0.15 ^a	0.07
160 (2X)	4.74 ^b	1.38 ^b	0.13 ^a	0.07
<i>P</i> value	0.0084	0.0033	0.0005	0.4714
SEM	0.78	0.50	0.02	0.01
Time of treatment (day)				
-1 (1 d pre-nitroethane treatment)	7.44 ^{a,b}	3.11	0.04 ^b	0.06
1	6.26 ^{a,b}	1.66	0.12 ^a	0.06
2	5.16 ^c	2.25	0.11 ^a	0.06
7	8.50 ^{b,c}	2.44	0.14 ^a	0.08
14 (at end of nitroethane treatment)	7.82 ^{b,c}	1.61	0.15 ^a	0.08
<i>P</i> value	0.0028	0.1184	< 0.0001	0.2698
SEM	0.62	0.45	0.02	0.01
Interaction				
<i>P</i> value	0.1797	0.9376	0.0003	0.8046
SEM	1.08	0.77	0.03	0.02

^{a, b, c}Values within columns with unlike superscripts differ ($P < 0.05$).

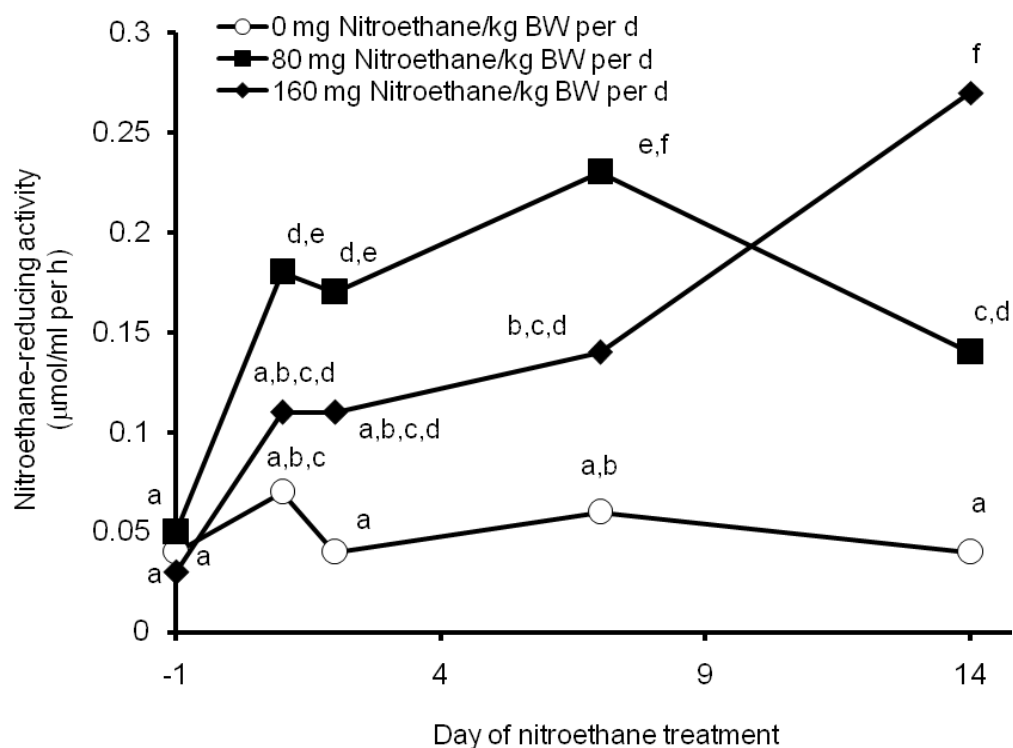


Figure 3.1. Effects of oral nitroethane administration on ruminal nitroethane-reducing activity in fed steers. Nitroethane was administered twice daily (08:00 and 16:00) via oral gavage. A repeated measures analysis of variance revealed a treatment by day interaction ($P = 0.0003$; $\text{SEM} = 0.03$), means with unlike superscripts differ at $P < 0.05$.

for 0X, 1X and 2X treatments, respectively), were not affected ($P > 0.05$) by nitroethane treatment. Daily DMI and ADG were not affected ($P > 0.05$) by treatment and averaged (\pm SE) 15.0 ± 0.5 kg/d and 1.28 ± 0.20 kg/day, respectively, over the 14 d nitroethane treatment period. An effect of day of treatment ($P < 0.05$), but not a treatment by day interaction was observed for whole animal CH_4 emissions. Methane emissions were lower on day 14 ($3.8 \pm 0.23\%$ GEI) of the study compared to day 7 ($4.3 \pm 0.23\%$ GEI).

Ruminal and Fecal Fermentation Balance

Acetate concentrations were lower ($P < 0.05$) in ruminal fluid collected from steers administered the 2X nitroethane treatment compared to steers administered the 0X or 1X nitroethane treatments (Table 3.3). Propionate and butyrate concentrations, as well as the ratio of acetate to propionate were unaffected by nitroethane treatment (Table 3.3). Estimates of CH_4 production derived from a fermentation balance tended to be lowest ($P < 0.07$) in ruminal fluid from steers administered the 2X nitroethane treatment than in fluid collected from steers administered the 0X and 1X nitroethane treatments (Table 3.3). Fecal VFA accumulations or estimated fecal CH_4 production were not affected ($P > 0.05$) by nitroethane treatment (Table 3.3).

An effect of day of nitroethane treatment was observed ($P < 0.05$) on ruminal VFA concentrations, with concentrations as well as the acetate to propionate ratio and estimated CH_4 production generally being higher on d 7 and 14 of nitroethane treatment than earlier days of the study (Table 3.3). In contrast, fecal acetate concentrations were lowest ($P < 0.05$) on d 2, 7 and 14 of nitroethane treatment and fecal propionate was

Table 3.3. Effect of oral nitroethane administration on ruminal fermentation balance in fed steers

Treatment (g nitroethane/kg BW per d)	Ruminal concentrations ($\mu\text{mol/g}$ contents)				Ratio of acetate to propionate
	Acetate	Propionate	Butyrate	Estimated methane	
0 (0X)	45.53 ^a	18.83	10.44	23.3	2.55
80 (1X)	42.96 ^a	18.76	12.25	22.9	2.39
160 (2X)	34.61 ^b	15.60	11.38	19.1	2.17
<i>P</i> value	0.0169	0.1515	0.3257	0.0627	0.1491
SEM	2.54	1.00	0.74	1.34	0.12
Time of treatment (day)					
-1 (1 d pre-nitroethane treatment)	24.42 ^c	15.77 ^b	9.20 ^b	12.8 ^b	1.68 ^c
1	35.91 ^b	17.15 ^{a,b}	9.91 ^b	18.6 ^b	2.14 ^{b,c}
2	37.51 ^b	15.62 ^b	10.36 ^b	20.0 ^b	2.51 ^{a,b}
7	55.66 ^a	19.70 ^a	14.52 ^a	30.2 ^a	2.81 ^a
14 (at end of nitroethane treatment)	51.67 ^a	20.41 ^a	12.82 ^a	27.1 ^a	2.7 ^a
<i>P</i> value	< 0.0001	0.0200	0.0002	< 0.0001	0.0005
SEM	2.98	1.28	0.88	1.70	0.19
Interaction					
<i>P</i> value	0.2374	0.2280	0.6166	0.3446	0.5503
SEM	5.50	2.36	1.63	3.14	0.35

^{a, b, c}Values within columns with unlike superscripts differ ($P < 0.05$).

lowest ($P < 0.05$) on d 2 and highest ($P < 0.05$) on d 14 of treatment (Table 3.4). Fecal butyrate concentrations were unaffected ($P > 0.05$) by day of treatment but the ratio of acetate to propionate was lowest ($P < 0.05$) on d 14 of treatment and the amount of estimated CH₄ produced was lowest on days 2 and 7 of treatment (Table 3.4). Treatment by day of treatment interactions were not observed ($P > 0.05$) for ruminal or fecal VFA accumulations, ratios of acetate to propionate or estimated CH₄ production.

Discussion

In agreement with earlier reports (Anderson et al., 2002; Anderson et al., 2005a; Callaway et al., 2002; Fox et al., 2005), results from the present study demonstrate that feeding an experimental chlorate product reduced ($P < 0.05$) generic *E. coli* concentrations in feces > 1,000-fold by 24 h post-chlorate treatment (Table 3.1). Moreover, these results provide additional information pertaining to the persistence of the bactericidal effect of chlorate: *E. coli* concentrations were reduced in the lower gut by 48 h post-chlorate treatment, albeit only 10-fold lower than pre-treatment concentrations (Table 3.3). This diminishing effect of chlorate over time is not unexpected; however, as chlorate exerts its effects by being catalytically reduced, and thus depleted, by membrane bound respiratory nitrate reductase (Nar), possessed by bacteria such as *E. coli* and *Salmonella* (Moreno-Vivian et al., 1999). No effect of the experimental chlorate treatment ($P > 0.05$) was observed on ruminal *E. coli* concentrations although this was expected as the chlorate product had been reported to possess rumen bypass characteristics (Edrington et al., 2003).

Table 3.4. Effect of oral nitroethane administration on fecal fermentation balance in fed steers

Treatment (g nitroethane/kg BW per d)	Fecal concentrations ($\mu\text{mol/g}$ contents)				Ratio of acetate to propionate
	Acetate	Propionate	Butyrate	Estimated methane	
0 (0X)	59.08	25.72	20.09	33.2	2.39
80 (1X)	67.69	29.17	20.45	36.8	2.51
160 (2X)	58.24	25.82	19.89	32.6	2.33
<i>P</i> value	0.2739	0.5658	0.9799	0.4493	0.6212
SEM	4.36	2.37	2.03	2.44	0.08
Time of treatment (day)					
-1 (1 d pre-nitroethane treatment)	76.51 ^a	30.62 ^{a,b}	20.54	40.9 ^a	2.64 ^a
1	64.11 ^{a,b}	25.11 ^{b,c}	20.07	35.8 ^{a,b}	2.56 ^a
2	52.05 ^b	21.26 ^c	16.97	29.2 ^b	2.50 ^a
7	54.67 ^b	25.09 ^{b,c}	19.94	31.0 ^b	2.33 ^{a,b}
14 (at end of nitroethane treatment)	61.02 ^b	32.43 ^a	23.20	34.0 ^{a,b}	2.01 ^b
<i>P</i> value	0.0079	0.0189	0.1296	0.0271	0.0070
SEM	4.92	2.59	1.67	2.65	0.13
Interaction					
<i>P</i> value	0.9380	0.7845	0.9712	0.9924	0.4146
SEM	9.08	4.78	3.08	4.89	0.24

^{a, b, c}Values within columns with unlike superscripts differ ($P < 0.05$).

Unlike that observed with swine, where an additive *E. coli*- and *Salmonella*-killing effect was observed with combined or prior addition of nitroethane with chlorate (Anderson et al., 2006b; Anderson et al., 2007), no interaction between chlorate and nitroethane was observed on generic *E. coli* populations in this study. Furthermore, unlike that observed earlier (Horrocks et al., 2005; Jung et al., 2003), nitroethane treatment in the present study had no effect against *E. coli* or *Campylobacter*. In those earlier studies, inhibition of *E. coli*, *Salmonella* or *Campylobacter* in swine gut contents was consistently observed at concentrations > 10 mM. Consequently, we suspect that the nitroethane dose administered in this study was too low (approximately 8.6 mM ruminal nitroethane concentration based on the 2X dose and a 100 liter rumen volume) to exert inhibitory activity against these bacteria. Alternatively, it is probable that some of the nitroethane may have been absorbed across the rumen wall, expired in eructated gases or consumed by gut bacteria such as the highly competent nitro-respiring *Denitrobacterium detoxificans* (Anderson et al., 2000c) thus further depleting gut concentrations of this inhibitor. In support of the later hypothesis, ruminal nitroethane-reducing activity increased markedly soon after initiation of nitroethane administration (Figure 3.1) and this likely contributed to depletion of nitroethane. No conclusions can be made regarding effects of nitroethane or experimental chlorate treatment on *E. coli* O157:H7, which were not detected pretreatment, or on the incidence of *Salmonella*, which was reduced 80 to 100% from pre-treatment measurements regardless of treatments.

In agreement with results of a previous study (Anderson et al., 2006a), oral nitroethane administration reduced ($P < 0.05$) ruminal methane-producing activity in this study (Table 3.2) and tended ($P < 0.10$) to reduce the theoretical production of ruminal CH_4 as estimated by fermentation balance. Ruminal methane-producing activity, which is an indirect measure of numbers of methanogens, and theoretical ruminal CH_4 production were shown to be correlated (Pearson correlation coefficient = 0.3513, $P = 0.001$). In contrast to earlier results (Brown et al., 2005), however, whole animal CH_4 emissions were not affected ($P > 0.05$) by nitroethane treatment in this study, possibly due to the high variability in CH_4 recovery and dry matter intake that was observed. As reported by others (Wright et al., 2004a), high day to day variation within and between animals and problems with missing measurements were encountered in this study, problems that when compounded by the low numbers of experimental units may have limited the ability of the method to detect potential treatment effects. Moreover, pretreatment emission measurements were lower for control steers than for 1X or 2X treated steers (Figure 3.2) which further confounded the results. Additionally, no correlation was found between sulfur hexafluoride-derived whole animal CH_4 emissions (%GEI) and ruminal methane-reducing activity (Pearson correlation coefficient = -0.1897, $P = 0.1965$) or theoretical production of ruminal CH_4 (Pearson correlation coefficient = -0.0562, $P = 0.7046$). Wright et al. (2004a) found no correlation between indirect chamber calorimetry measurements of whole animal CH_4 emissions and those obtained using the sulfur hexafluoride method. An effect of day of treatment ($P < 0.05$)

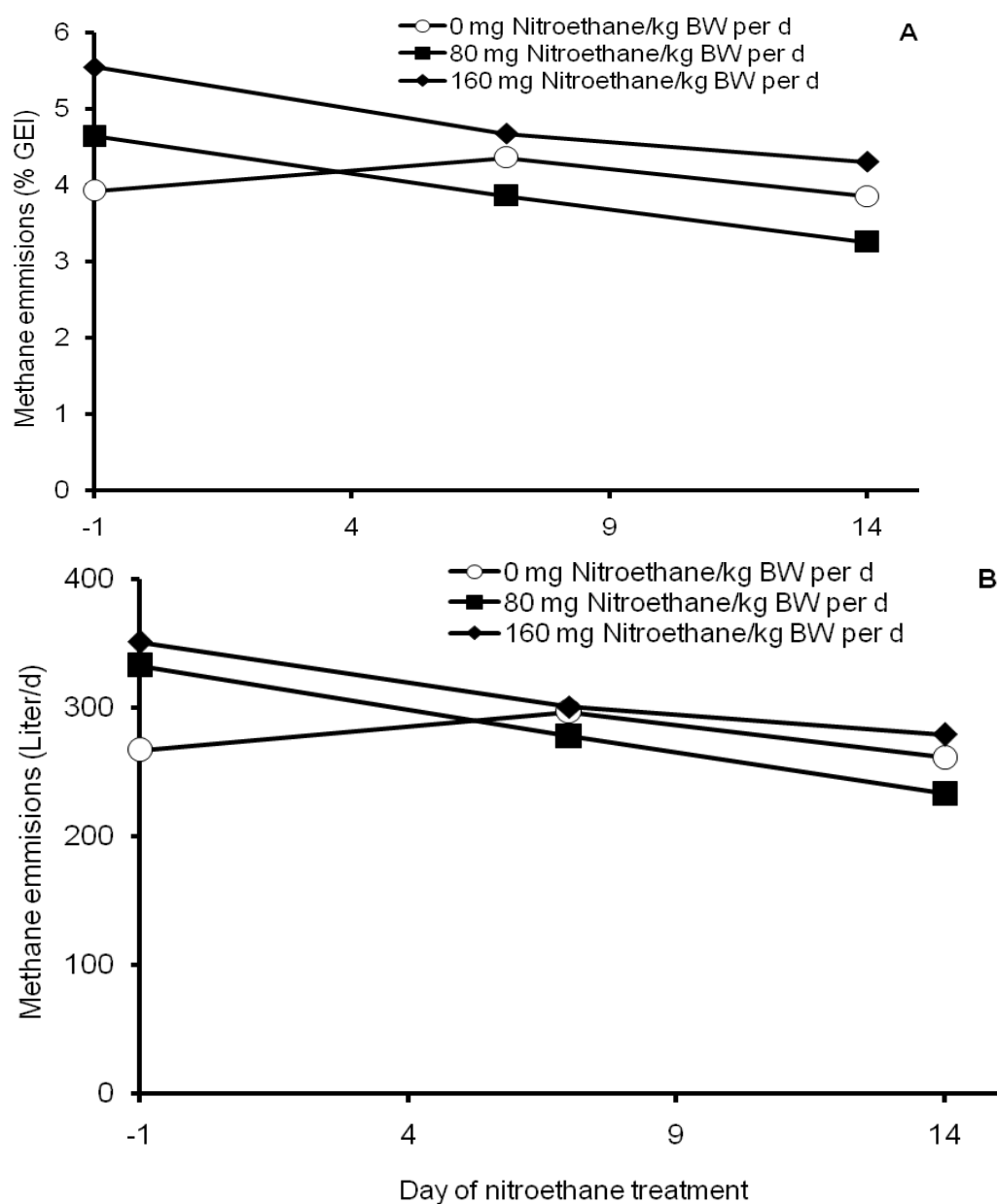


Figure 3.2. Effects of oral nitroethane administration on ruminal methane emissions, when expressed as percent of gross energy intake (A) or liters/d (B), in fed steers. Nitroethane was administered twice daily (08:00 and 16:00) via oral gavage. A repeated measures analysis of variance revealed no treatment by day interaction ($P = 0.3447$ and 0.4157 for A and B, respectively; $SEM = 0.41$ and 29.34 , respectively).

was observed on whole animal CH₄ emissions, with the lowest production occurring on day 14 compared with that produced pretreatment or on day 7. A day effect was also observed on methane-producing activity, but in this case reductions in activity diminished over time, suggesting the occurrence of an apparent adaptation within the rumen ecosystem. Ruminal adaptation to CH₄ inhibitors is well known (Van Soest and Demeyer, 1996). In the present study, the adaptation was mainly due to a transient decrease in methane-producing activity in ruminal contents from steers administered the 1X nitroethane treatment as the methane-producing activity in contents from steers administered the 2X treatment remained at least 30% lower than the activity measured pre-treatment (Figure 3.3). An apparent adaptation was observed in the earlier study as well as in sheep administered 24 or 72 mg nitroethane/kg BW per day (Anderson et al., 2006a). Adaptation likely occurs, at least in part, because of an enrichment of ruminal nitroethane-consuming bacteria as evidenced in the present study by main effects of nitroethane treatment, day of treatment (Table 3.2) and their interaction on ruminal nitroethane-reducing activity (Figure 3.1). At present, *D. detoxificans*, an obligate nonfermentative nitro-respiring anaerobe, is the only ruminal bacterium known to possess appreciable ability to metabolize nitroethane, as well as a variety of other oxidized nitrocompounds, coupling their reduction to the oxidation of H₂, formate or lactate (Anderson et al., 2000c). It is known that concentrations of this bacterium can be increased >1000-fold and rates of nitrocompound metabolism can be increased during growth with additions of a related nitrocompound, 3-nitro-1-propanol, the poisonous compound found in various species of the leguminous forage *Astragalus* (milkvetches)

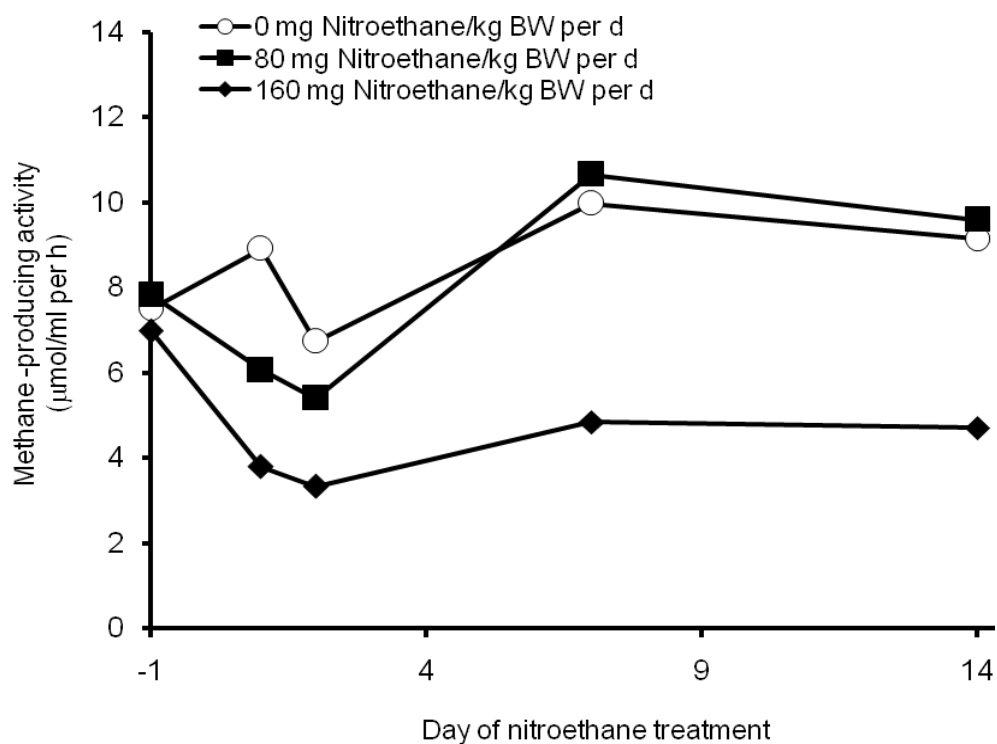


Figure 3.3. Effects of oral nitroethane administration on ruminal methane-producing activity in fed steers. Nitroethane was administered twice daily (08:00 and 16:00) via oral gavage. A repeated measures analysis of variance revealed no treatment by day interaction ($P = 0.1797$; SEM = 1.08).

(Anderson et al., 1996). Supplementing cattle diets with nitroethane also increased disappearance rates of ruminal 3-nitro-1-propanol (Majak, 1992; Majak et al., 1986). In the present study, ruminal nitroethane-reducing activity increased to $> 0.17 \pm 0.05 \mu\text{mol}$ nitroethane/mL per h for steers administered the 1X nitroethane treatment indicating that more than 90% of their daily dose (estimated to be $4.3 \mu\text{mol}$ nitroethane/mL ruminal fluid per day) would have been consumed by 24 h. Thus, it is reasonable to expect that while effective methane-inhibiting concentrations of nitroethane may have been maintained in the steers administered the 2X nitroethane treatment, concentrations were probably depleted in the steers administered the 1X treatment. Results from *in vitro* incubations of ruminal contents have shown that the methane-inhibiting effect of nitroethane was reduced approximately 36% when nitroethane concentration was reduced from 12 to 2 mM (Anderson et al., 2003).

Many CH_4 inhibitors that directly inhibit methanogenic bacteria dissipate the H_2 consuming role played by methanogens. This subsequently results in decreased accumulations of acetate and, as a compensatory route for dispensing of reducing equivalents, increased accumulations of more reduced fatty acids such as propionate and butyrate (Van Nevel and Demeyer, 1996). While the mechanistic effects of nitroethane on methanogens has yet to be determined, a direct chemical inhibition is likely, at least initially, as related compounds, 3-nitro-1-propionic acid and 3-nitro-1-propanol, were shown to inhibit *Methanobrevibacter ruminantium* and *Methanobrevibacter smithii* directly (Anderson, 1998). In this and an earlier study (Anderson et al., 2006a), however, administration of nitroethane, or a related compound, 2-nitro-1-propanol, at less than 80

mg/kg BW per day had no effect on ruminal accumulation of volatile fatty acids. Acetate accumulation was decreased ($P < 0.05$) in ruminal contents from sheep administered 120 mg 2-nitro-1-propanol (Anderson et al., 2006a) and in the steers in this study administered the 2X nitroethane treatment thus suggesting a potential detrimental effect of these higher doses. It is unlikely that nitroethane caused inhibitory accumulations of H_2 within the rumens of nitroethane-treated steers as reducing equivalents appeared not to be redirected to the production and accumulation of the more reduced acids, propionate and butyrate, which were unaffected ($P > 0.05$) by treatment (Table 3.3). This conclusion is further supported by the observation that the ratio of acetate to propionate was unaffected ($P > 0.05$) by nitroethane treatment (Table 3.3). Moreover, only small quantities of H_2 ($< 3 \mu\text{mol/mL}$ ruminal fluid) were shown to accumulate in vitro incubations of ruminal fluid with nitroethane, 2-nitro-1-propanol or 2-nitroethanol (Anderson et al., 2003). The reduction of nitroethane would be expected to consume at least some of the reducing equivalents not used to reduce CO_2 to CH_4 and as nitroethane-reducing activity increased more equivalents would be consumed. Thus, while nitrocompounds may initially exert a direct inhibition on methanogenic bacteria, inhibition of CH_4 production could also progress to be partly competitive, as numbers of hydrogen-oxidizing bacteria like *D. detoxificans* increase, they may be able to outcompete methanogens for reducing equivalents. In vitro incubation of *D. detoxificans* strain NPOH1 in ruminal contents with added nitrate as an electron acceptor inhibited CH_4 production by 94% (Anderson, 1998). Reducing equivalents may also be consumed to support anabolic cell processes and growth of increasing populations of nitroethane-

reducing bacteria. In contrast to that observed with ruminal fluid, in vitro incubations of chicken cecal contents with nitroethane resulted in appreciable accumulations of H₂ (more than > 10 µmol hydrogen/g cecal content) over that produced by of that of control cultures incubated without nitroethane (Saengkerdsub et al., 2006). It is not known whether or not chickens may be colonized with *D. detoxificans*, which if absent, could possibly explain why there was no apparent alternative H₂ sink. Presently, *D. detoxificans* has only been isolated from ruminal contents (Anderson et al., 1996; Anderson et al., 2000c) although *D. detoxificans*-like nucleic acid has been recovered from human dental caries (Chhour et al., 2005).

As in an earlier study (Anderson et al., 2006a) ruminal fermentation efficiency appeared to increase during the study's progression as evidenced by an increase in ruminal VFA accumulations over time for all steers (Table 3.3) and this was coincident with decreases in fecal acetate and propionate (albeit transient) concentrations (Table 3.4). It is possible that this may have due to the buffering capacity of the alkaline (pH 10.2) phosphate buffer used as a placebo or carrier of nitroethane; however, this is unlikely as total daily amounts of buffer or treatment additions did not exceed 360 mL which, assuming a rumen volume of 100 L, is less than 0.4% total ruminal volume. The alkaline buffer may have affected increased ruminal digestion thereby decreasing substrate passage to the lower gut. If this is the case, then decreased substrate availability, rather than nitroethane per se, may be responsible for the decreased methane-producing activity observed in fecal contents of the nitroethane-treated steers in this study. Fecal nitroethane-reducing activity did not differ between control- and

nitroethane-treated steers, but this was more likely due to reduced access to lower concentrations of nitroethane.

Conclusions

Results demonstrated that nitroethane administration reduced methane-producing activity, an indirect measure of numbers of methane-producing bacteria, in rumen contents of growing steers fed the mixed diet by more than 40% although these results were not corroborated by measurements of sulfur hexafluoride determination of whole animal CH₄ emissions. Contrary to findings from numerous in vitro and in vivo studies in swine, our results did not support the hypothesis that nitroethane would reduce *Campylobacter* and *Salmonella* in these fed steers, possibly because rapid absorption, expiration and rumen degradation prevented accumulations of nitroethane to levels needed to be effective against these enteropathogens. Studies testing the related nitrocompounds, 2-nitroethanol and 2-nitro-1-propanol, which exert greater anti-*E. coli*, anti-*Salmonella* and anti-*Campylobacter* activity than nitroethane (Anderson et al., 2007; Horrocks et al., 2005) as alternatives to nitroethane may be warranted, although in the case of 2-nitro-1-propanol, its methane-inhibiting activity was found to be inferior to that exerted by nitroethane (Anderson et al., 2006a). Feeding chlorate in the last day's meal reduced ($P < 0.05$) fecal *E. coli* concentrations by up to 1000-fold but this was not enhanced by prior nitroethane treatment. Because an apparent microbial resistance could be operative, further work is needed to determine if dosage of nitroethane or related nitrocompounds can be optimized to achieve and maintain concomitant enteropathogen control and CH₄ reduction in fed steers.

CHAPTER IV
NITROETHANE AND MONENSIN EFFECTS ON RUMINAL FLUID
FERMENTATION

Introduction

Methane (CH_4) is a reduced end product of ruminal fermentation processes and contributes to the rumen microbial ecology by maintaining a low partial pressure of hydrogen (H_2) thus allowing fermentation to proceed unencumbered by facilitating reduced nucleotide re-oxidation by hydrogenase activity rather than by alcohol- or lactate-dehydrogenases (Russell and Wallace, 1988). However, there are negative aspects of ruminant methanogenesis. Methane is considered a loss of gross energy intake (2-15%) of the total feed consumed by the whole animal (Van Nevel and Demeyer, 1996) and its inhibition is directly related to improvements in feed efficiency. Also, there is the increasing concern that global climate is being changed due to the accumulation of greenhouse gases (Harper et al., 2007; UN, 1998) and CH_4 is considered a highly potent greenhouse gas (UN, 1998) because of its ability to absorb infrared radiation from the sun (Mohammed et al., 2004). Many attempts to decrease CH_4 production have been evaluated, and one of the most prominent research fields to decrease CH_4 production is testing for alternative electron sink acceptors for H_2 (Moss et al., 2000). Efforts using organic acids, such as fumarate (McGinn et al., 2004), aspartate and malate (Callaway and Martin, 1996), or oxaloacetate (Ungerfeld et al., 2003) have been tested. Also, butyrate precursors, crotonic acid and 3-butenic acid, which were ineffective as

electron acceptors (Ungerfeld et al., 2006). However, organic acids would have limited usage until either costs decrease or greenhouse gas credits increase dramatically (Newbold, 2005). Other attempts using ionophores such as monensin, with its concomitant change in the acetate:propionate ratio and rumen fermentation parameters, have been proposed as CH₄-reducing strategies (Hristov et al., 2003; Tedeschi et al., 2003). However, public health concerns regarding use of agricultural antibiotics that may lead to the development of antimicrobial resistance has resulted in restrictions on the use of monensin in the European Union. Furthermore, an increase in demand for organically produced foods may ultimately restrict the use of ionophores in the United States (Russell and Houlihan, 2003).

Nitroethane has been safely administered to cattle and has the potential to serve as an alternative electron acceptor within the rumen (Anderson et al., 2003; Majak et al., 1986). Moreover, it is likely that nitroethane may also exerts direct chemical-inhibition of methanogens as was observed with a closely related compound, 3-nitro-1-propionic acid (Anderson, 1998). Nitroethane has been shown to decrease CH₄ production in vitro, with little effect on molar proportions of volatile fatty acids (VFA); thereby, maintaining the fermentation balance (Anderson et al., 2003), and in vivo, although dose-effect and possible ruminal adaptation to low doses were reported (Anderson et al., 2006a; Gutierrez-Banuelos et al., 2007). Whereas these studies have provided information on the effect of nitroethane on some ruminal fermentation characteristics, aspects dealing with ruminal adaptation to nitroethane, particular by nitro-metabolizing bacteria, remain unclear. The objectives of this study were to evaluate and compare the effects of

nitroethane and monensin on ruminal fermentation and nitro-metabolizing bacteria populations.

Material and Methods

Experimental Design

Ruminal fluid was collected into a pre-heated thermos from a ruminally fistulated Holstein-Friesian steer grazing rye grass (*Lolium perenne*). Ruminal contents were squeezed through a nylon paint strainer (Leyendecker et al., 2004) to obtain particle-free rumen fluid. The pH of the rumen fluid before delivery into tubes was 6.72. Basal medium, which was prepared and inoculated aseptically using anaerobic technique (Paynter and Hungate, 1968), was supplemented with 0.2 g of ground alfalfa and treated with distilled water (C), 4.5 mM/mL nitroethane (1NE), 9 mM/mL nitroethane (2NE), 5 mM/mL monensin (M), and 9 mM/mL nitroethane plus 5 mM/mL monensin (2NEM). Stock solutions of nitroethane were prepared as the sodium salt (Majak et al., 1986) and monensin was diluted in ethanol (Callaway and Martin, 1996). A consecutive batch culture (CBC) technique (Theodorou et al., 1987) was started by inoculating three replicate culture tubes for each treatment with 1 mL of the particle-free rumen fluid. After 24 h of incubation (39 °C) under H₂:CO₂ (1:1), 1 mL samples were used to inoculate the next series of anaerobic culture tubes, which contained fresh treated medium. A total of 16 series were conducted in this way, by sequential transfer of inoculum from the previous series of tubes to fresh medium. Methane, H₂ and carbon dioxide (CO₂) were measured on series 1, 2, 3, 6, 10, 13 and 16. Ammonia, net VFA production, lactate and nitroethane-concentration were measured on series 1, 2, 3, 6 and

10. Net VFA production was calculated as the difference between the start-time (0 h) and the end-time (24 h) for each incubation series.

The most probable number (MPN) method using series of three tubes (AOAC., 1980) was used at the sixth series of the experiment to quantify nitro-metabolizing bacterial populations. The culture medium was prepared employing medium B (Anderson et al., 1996) supplemented with 6 mM of 3-nitro-1-propionic acid (NPA). After 14 days of incubation (39 °C) under anaerobic conditions (H:CO₂; 1:1 gas phase), the remaining NPA was quantified and considered positive for nitrocompound-reducing bacteria if more than 50 % of the 6 mM of the NPA was metabolized. 3-Nitro-1-propionic acid was used as the test substrate because it is less volatile than nitroethane and like nitroethane, is metabolized in appreciable amounts exclusively by the obligate respiratory ruminal anaerobe *Denitrobacterium detoxificans* (Anderson et al., 2000c).

In a final experiment, the treatments were reversed to provide a cross over experiment. From each treatment, two replicates were chosen at the sixteenth series, and were inoculated to test all treatments. After 24 h incubation period, CH₄, H₂ and CO₂ were measured.

Analytical Procedures

Concentrations of H₂, CH₄ and CO₂ were measured via gas chromatography from samples collected on the headspace of each tube (Allison et al., 1992; Anderson, 1998). Volatile fatty acids, specifically acetate, propionate, butyrate, valerate, isobutyrate and isovalerate, were determined from the fluid contents using gas chromatography (Hinton et al., 1990). Concentrations of nitrocompounds and ammonia were determined

colorimetrically (Chaney and Marbach, 1962; Majak et al., 1986). Estimates of CH₄ and CO₂ were derived from the fermentation balance equation (Wolin, 1960).

Statistical Analysis

Multiple comparisons of LSM were obtained by treatment, adjusted by Tukey and expressed significant if $P < 0.05$. Contrasts within series were calculated to assess treatment differences ($P < 0.05$) on CH₄, CO₂, H₂, acetate, propionate, and butyrate. Orthogonal polynomial contrasts, specifically the linear and quadratic main effects, were computed within treatment. All data were analyzed using PROC MIXED SAS 9.1.3. Pearson correlation coefficients for all variables were calculated within series using PROC CORR SAS 9.1.3.

Results

Methane, Hydrogen and Carbon Dioxide

An effect of treatment was found on CH₄ production ($P < 0.001$). The addition of both nitroethane and monensin decreased CH₄ production (μmol/mL) in comparison to the C treatment. However, treatments with nitroethane showed a stronger antimethanogenic effect in comparison with monensin alone (8.48, 0.88, 0.81, 1.88, and 0.74 ± 0.37 SEM; respectively for C, 1NE, 2NE, M, and 2NEM treatment) (Figure 4.1). No effect of treatment ($P > 0.05$) was observed on H₂ concentration (μmol/mL) averaging 25.34, 24.25, 23.71, 22.48, and 21.74 ± 1.40 SEM; respectively for C, 1NE, 2NE, M, and 2NEM treatment (Figure 4.1). Carbon dioxide (μmol/mL) was lower ($P < 0.01$) in the 2NEM in comparison with C (67.78 and 99.94, respectively), and no

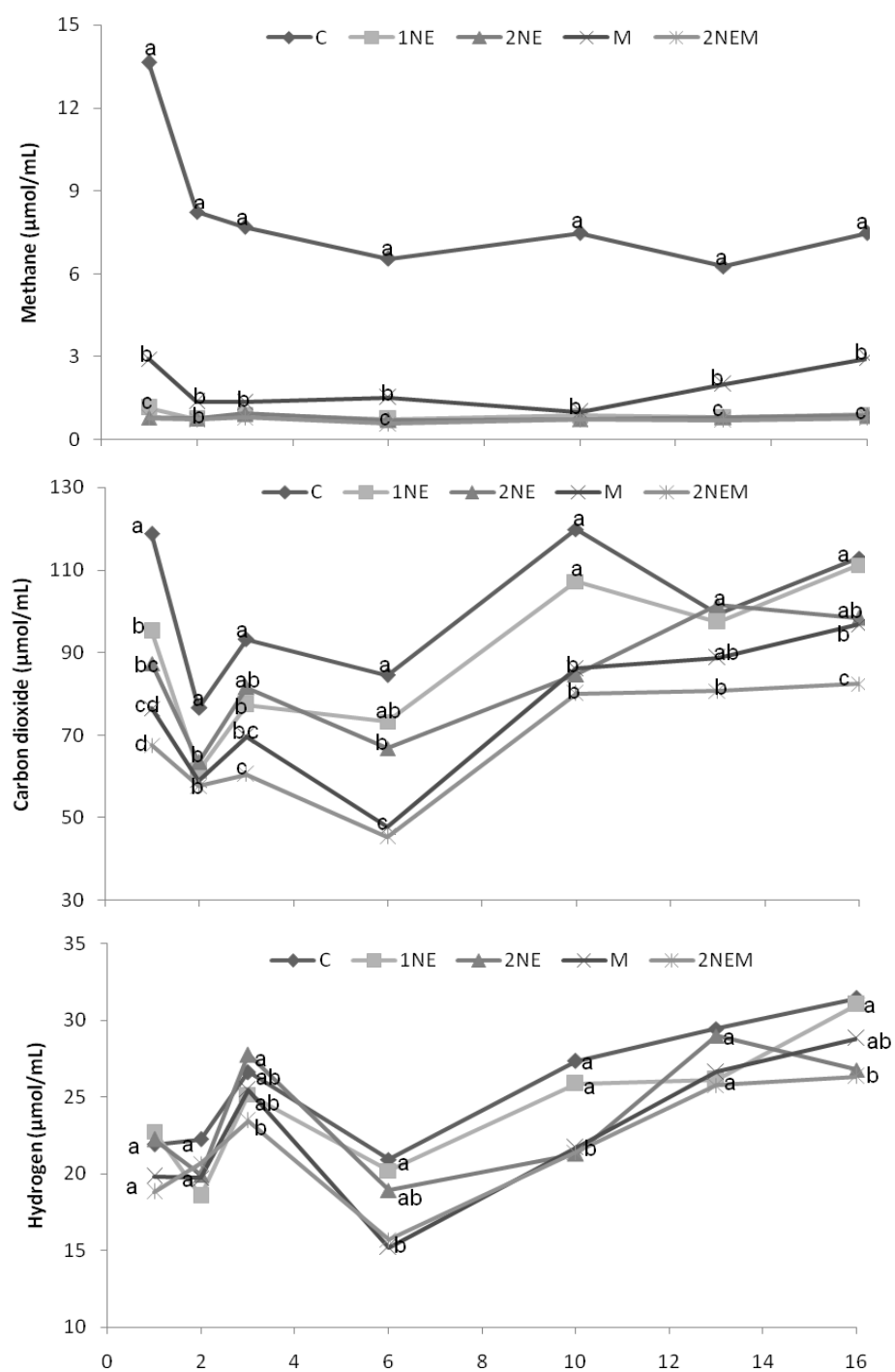


Figure 4.1. Effect of nitroethane and monensin on methane, carbon dioxide and hydrogen production on a consecutive batch culture. ^{abc}Values within series with unlike superscript differ ($P < 0.05$).

differences were observed among 1NE, 2NE and M treatments (89.00, 83.37, and 74.93 \pm 5.30, respectively) in comparison to the C treatment (Figure 4.1).

Lactate and Ammonia

Lactate and ammonia concentrations were affected by treatment ($P < 0.001$) (Table 4.1). Lactate levels (mg/dL) decreased with 2NE in comparison to the C treatment (13.93 and 16.91 \pm 0.75 respectively). Ammonia concentrations ($\mu\text{mol/mL}$) showed the lowest levels with 1NE and M in comparison to the C (2.63, 2.65, and 3.01 \pm 0.12 respectively).

Volatile Fatty Acids, Estimated Methane and Estimated Carbon Dioxide

Neither acetate (25.41, 25.81, 30.03, 21.00, and 17.04 \pm 4.70 $\mu\text{mol/mL}$) nor propionate (13.26, 13.63, 14.86, 13.04, and 12.30 \pm 1.40 $\mu\text{mol/mL}$) production, respectively, with C, 1NE, 2NE, M, and 2NEM, were affected by treatment ($P > 0.05$). An effect of treatment ($P < 0.001$) was observed on butyrate accumulation ($\mu\text{mol/mL}$), with lower concentrations observed in M and 2NEM incubations and higher accumulations observed in 1NE and 2NE supplemented incubations in comparison with the C treatment (1.73, 1.89, 5.65, 6.10, and 4.81 \pm 0.73, respectively) (Figure 4.2). Average concentrations of valerate, isobutyrate, isovalerate and the CH_4 , CO_2 , and acetate:propionate ratio calculations are shown in Table 4.1. The acetate:propionate ratio was affected ($P < 0.05$) by 2NEM in comparison to C (1.29, and 1.90 \pm 0.20, respectively). However, the other treatments did not differ from that calculated for C. Valerate and isobutyrate accumulations ($\mu\text{mol/mL}$) were increased ($P < 0.01$) by 2NE in comparison to the C (1.68 and 1.02 \pm 0.22; 0.27 and 0.16 \pm 0.04; respectively for

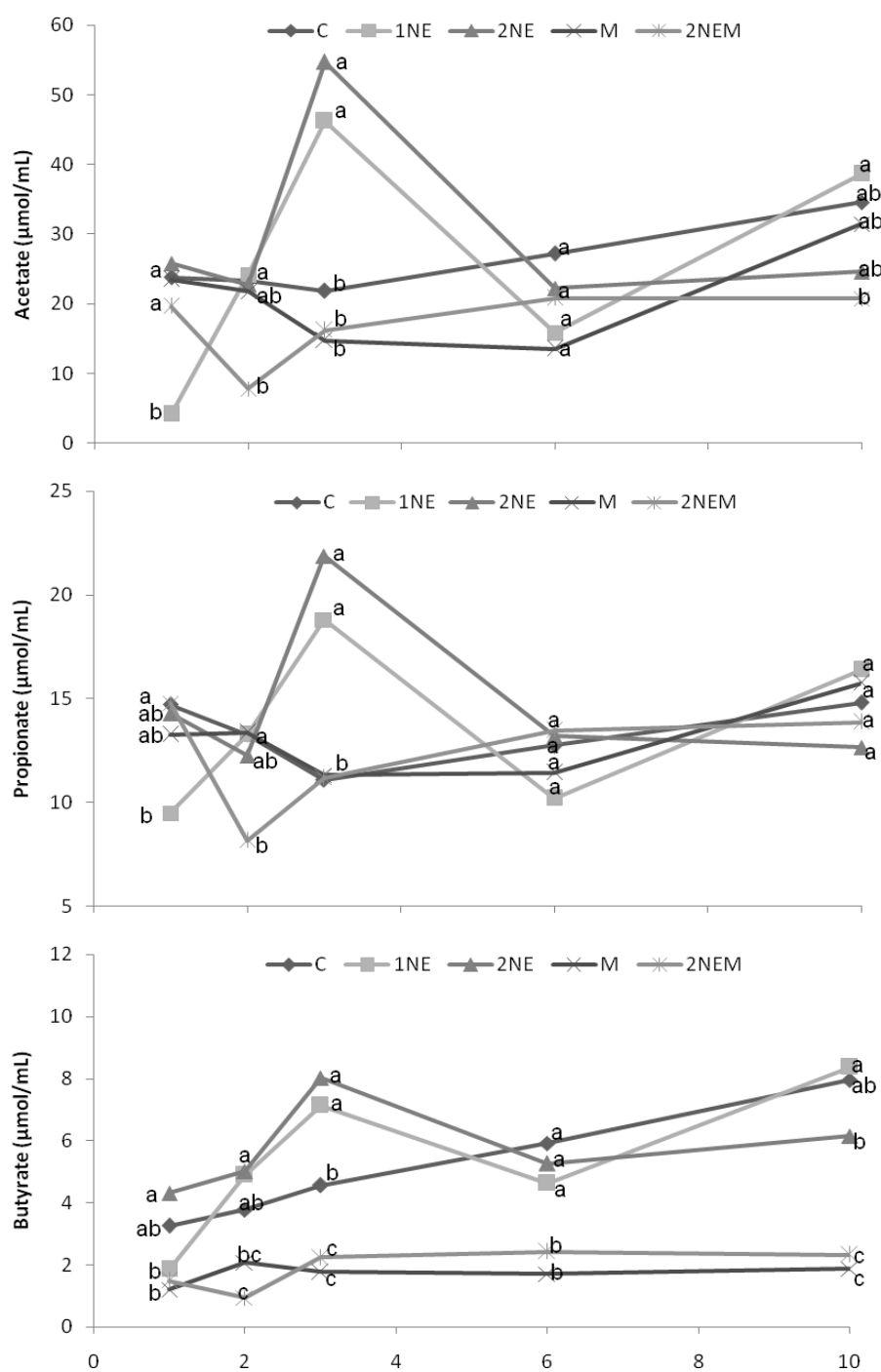


Figure 4.2. Effect of nitroethane and monensin on volatile fatty acid concentration on a consecutive batch culture. ^{abc}Values within series with unlike superscript differ ($P < 0.05$).

valerate and isobutyrate). Isovalerate concentration was not affected by treatment ($P > 0.05$). Theoretical production of CO_2 and CH_4 were not correlated ($P > 0.05$) with the respective values obtained from the tube headspace in any series.

Nitroethane-degradation and Nitro-metabolizing Bacterial Populations

Treatment effects of nitroethane-concentration are shown in Table 4.1.

Nitroethane-concentration ($\mu\text{mol/mL} \pm \text{SEM}$) was affected by treatment ($P < 0.01$), 2NEM showed more nitroethane-concentration in comparison with 1NE and 2NE-treatments ($1.26, 0.41, 0.95 \pm 0.06$; respectively), indicating that nitroethane-degradation was negatively affected by monensin.

The MPN of nitro-reducing bacteria were increased ($P < 0.01$) in 1NE and 2NE (6.9 and $5.9 \log_{10}$ MPN/mL, respectively) at the sixth incubation series compared to those in C, M and 2NEM treatments ($< 2.5 \log_{10}$ MPN/mL) (Figure 4.3 see page 61).

Crossover Experiment

When the treatments were reversed to provide a cross over experiment, the average of methane produced over all nitroethane treatments (1NE, 2NE, and 2NEM) were lower ($< 0.82 \mu\text{mol/mL}$; $P < 0.05$) in comparison to C and M treatments ($> 2.4 \mu\text{mol/mL}$) (Figure 4.4 see page 62).

Discussion

The consecutive batch culture (CBC) technique has been used to study mixed populations of ruminal microbes and their fermentative, fibrolytic, amylolytic and CH_4 producing activities in vitro. However, certain microbial populations, such as protozoa, do not survive (Theodorou et al., 1987). Protozoa release reducing equivalents (H_2)

Table 4.1. Effect of nitroethane and monensin on methane production and fermentation balance on a consecutive batch culture

Traits ($\mu\text{mol/mL}$)	Treatments					SEM [†]	<i>P</i> value
	C	1NE	2NE	M	2NEM		
Ammonia	3.01 ^a	2.63 ^b	2.85 ^{ab}	2.65 ^b	2.84 ^{ab}	0.12	0.01
Lactate*	16.91 ^a	14.83 ^{ab}	13.93 ^b	15.71 ^{ab}	16.38 ^a	0.75	0.001
NE-degradation [‡]	-	0.41 ^a	0.95 ^b	-	1.26 ^c	0.06	< 0.001
C2:C3 ratio	1.90 ^a	1.66 ^{ab}	1.92 ^a	1.55 ^{ab}	1.29 ^b	0.20	0.02
Valerate	1.02 ^{ac}	1.55 ^{ab}	1.68 ^b	0.40 ^c	0.59 ^c	0.22	< 0.001
Isobutyrate	0.16 ^a	0.20 ^{ab}	0.27 ^b	0.14 ^a	0.15 ^a	0.04	0.006
Isovalerate	0.10	0.12	0.15	0.12	0.12	0.03	0.55
Estimated CH ₄	11.80 ^{ab}	12.32 ^{ab}	14.35 ^a	8.10 ^{ab}	6.39 ^b	2.33	0.007
Estimated CO ₂	23.24 ^{abc}	24.78 ^{ab}	27.88 ^a	16.35 ^{bc}	14.42 ^c	3.73	0.002

^{a,b,c}Values within rows with unlike superscripts differ ($P < 0.05$)

* Lactate values expressed in mg/dL

[‡]Nitroethane-degradation

[†]Standard Error of the Mean

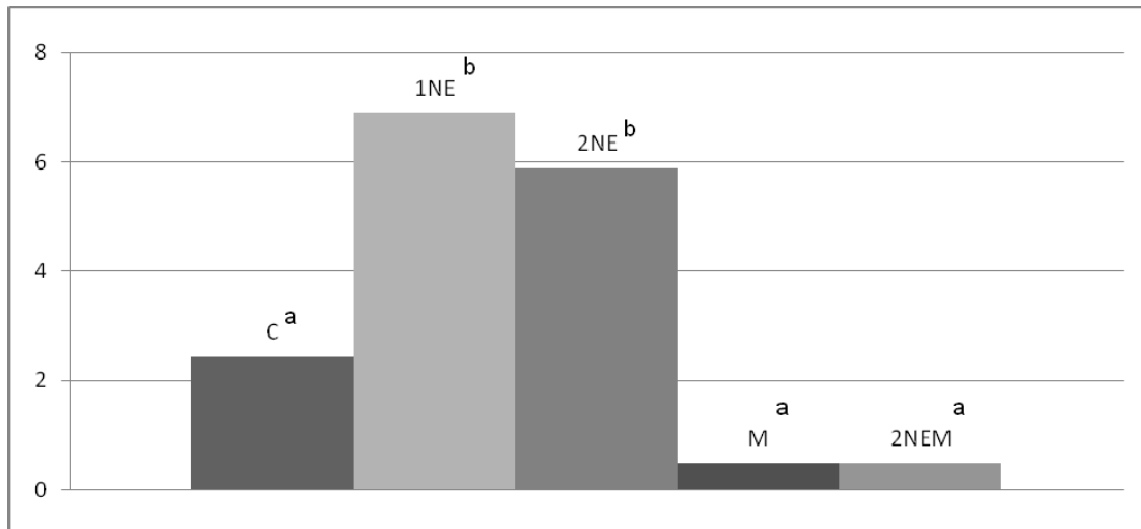


Figure 4.3. Effect of nitroethane and monensin on most probable number of nitro-metabolizing bacterial populations on a consecutive batch culture. ^{ab}Values with unlike superscript differ ($P < 0.05$).

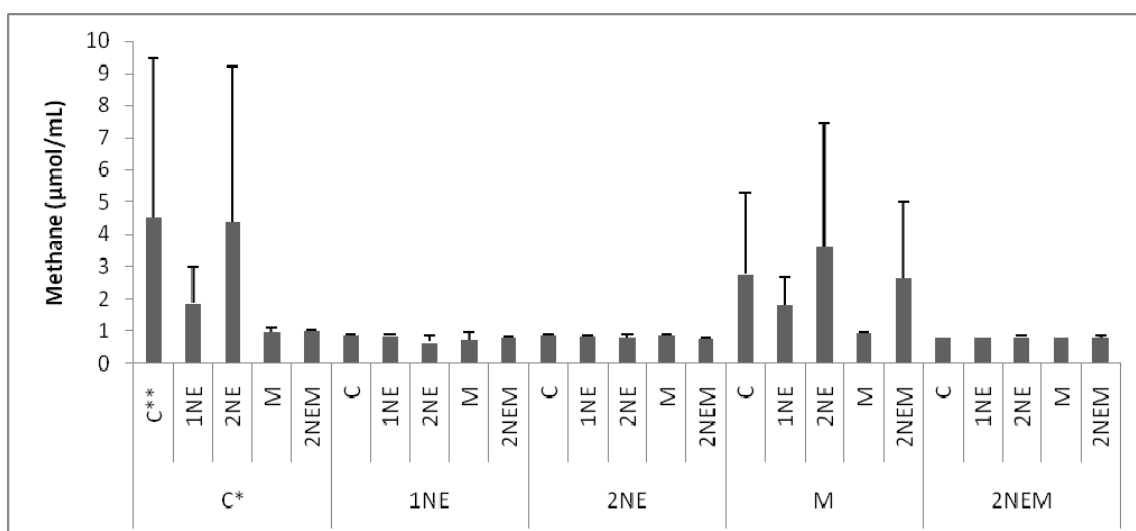


Figure 4.4. Effect of nitroethane and monensin on methane production after a cross over experiment on a consecutive batch culture. *Treated mediums after sixteenth batches.

**Treated mediums at the seventeenth batches. Error bars indicate SD.

(Tokura et al., 1997), which are used by methanogens to reduce CO_2 and form CH_4 (Miller, 1995). Protozoa are also associated with methanogens which can live on the exterior or as endosymbiotic organisms within the protozoa (Chagan and Kazunari, 2004). Therefore, loss of protozoa during in vitro incubation may explain the decrease in CH_4 production observed in this study after the first series for the C treatment (Figure 4.1).

Results from the present study confirmed the antimethanogenic effects of nitroethane and monensin, although inhibition of CH_4 production was greater in nitroethane supplemented incubations (1NE, 2NE and 2NEM) than in M (by as much as 90% versus 78%, respectively) when compared to the C treatment. The negative effect of nitroethane on CH_4 production was supported by a negative correlation in all series between CH_4 -production and nitroethane-concentration (Pearson correlation coefficient = -0.79, $P < 0.01$; -0.79, $P < 0.01$; -0.69, $P < 0.01$; -0.77, $P < 0.01$; and -0.71, $P < 0.05$, respectively for series 1, 2, 3, 6, and 10), which indicated that when nitroethane was metabolized, a decrease in CH_4 production was observed. Methane and CO_2 production were positively correlated (Pearson correlation coefficient) on series 1 and 2 (0.80 and 0.78, $P < 0.01$), 3 and 6 (0.65 and 0.64, $P < 0.05$), and tended to be correlated on series 10 (0.58, $P = 0.06$), which could be explained because CO_2 is the most important substrate for CH_4 production. Also, CO_2 had a quadratic effect ($P < 0.05$) on C, 1NE, M, and 2NEM and tended to have a quadratic ($P < 0.06$) behavior on the 2NE treatment. Theoretical CH_4 and CO_2 production were not correlated with the respective values obtained from the headspace. Probably due to the absence of other electron sink

acceptors in the stoichiometric equations, such as branched chain fatty acids, ethanol, and nitroethane per se.

Neither nitroethane or monensin, when administered individually, changed the amounts of acetate, propionate or the acetate:propionate ratio, which has been previously reported with nitroethane (Anderson et al., 2003; Anderson et al., 2006a). However, it is known that monensin typically changes the fermentation, resulting in a increased propionate accumulation at expense of CH_4 and a decreased molar proportion of acetate (Callaway et al., 1997; Nagaraja et al., 1997). The lack of propionate enhancing activity of monensin is likely due to the use of an $\text{H}_2:\text{CO}_2$ (1:1) gases environment in the CBC, which provided an abundant supply of reductant even for the control incubations. Typically, compounds that block reductive steps in methanogenesis in the rumen have induced a reduction of CH_4 and accumulation of H_2 (Garcia-Lopez et al., 1996) but neither monensin or nitroethane affected final accumulation of H_2 in the supplemented incubations when compared to accumulations in the control, which was approximately $24 \mu\text{mol/mL}$ incubation fluid. Again, this is likely due to the use of an $\text{H}_2:\text{CO}_2$ (1:1) gas phase. Results from an earlier study showed that propionate production was not increased and only small amounts of H_2 ($< 1.9 \mu\text{mol/mL}$ of rumen fluid) accumulated during in vitro incubations of ruminal fluid with nitroethane (Anderson et al., 2003) when 100% CO_2 was used as the gas phase. Therefore, in the case of nitroethane, alternative mechanisms of H_2 consumption, such as for reduction of nitroethane or for anabolic microbial synthesis, may have been detectable if H_2 is not in great excess. Butyrate was decreased by approximately 62% with the addition of monensin (M and

2NEM treatment) in comparison to the C treatment, likely because of the negative effect of monensin on butyric acid-producing bacteria, such as *Butyrivibrio fibrisolvens*, *Eubacterium cellulosolvens*, and *Eubacterium ruminantium* (Nagaraja et al., 1997). In contrast, nitroethane (1NE and 2NE treatments) increased butyrate concentrations by approximately 22% in comparison with the C treatment, which is contradictory with previous reports (Anderson et al., 2003; Anderson et al., 2006a). Branched chain fatty acids in ruminal contents arise from degradation of branched-chain amino acids (Wallace R. J., 1994) and so lower accumulations of branched chain amino acids observed during this study in the monensin-supplemented incubations suggest an inhibition of amino acid catabolism; however, enhanced utilization of branched chain fatty acids by bacteria cannot be ruled out. Our observation of increased accumulation of butyrate, valerate and isobutyrate in nitroethane-supplemented incubations compared to that in the monensin-supplemented cultures suggests an important mechanism of nitroethane activity that has not been previously reported.

Ammonia concentrations in the incubations were decreased on 1NE and M treatments by approximately 12% in comparison to the C treatment, which also suggests a decreased deamination rate and/or improved ammonia incorporation by rumen microorganisms. Monensin has been reported to negatively affect hyper-ammonia-producing organisms such as *Clostridium aminophilum*, *C. sticklandii* and *Peptostreptococcus anaerobius* (Nagaraja et al., 1997), as well inhibiting degradation of protein hydrolysates and consequently deamination (Russell and Strobel, 1989; Wallace et al., 1997). However, monensin is usually, but not always, associated with a decreased

amount of ammonia accumulation (Garcia-Lopez et al., 1996). Monensin has been reported to negatively affect lactic acid producer bacteria, as *Lactobacillus ruminis*, *Lactobacillus vitulinus*, and *Streptococcus bovis* (Nagaraja et al., 1997; Stewart and Bryant, 1988); however, lactate was not affected with the addition of this ionophore, but 2NE treatment decreased it.

Monensin, which is an antiporter with a high selectivity for sodium and with the ability to translocate potassium (Russell and Strobel, 1989), has been shown to be a highly lipophilic substance which is toxic to many bacteria, protozoa and fungi. In this experiment, it was observed that monensin negatively affected nitro-metabolizing bacteria. The most probable number of nitro-metabolizing bacteria tended to be lower ($P < 0.11$) on M and 2NEM in comparison with C, moreover the addition of NE as 1NE and 2NE increased ($P < 0.01$) the MPN of nitro-metabolizing bacteria in comparison with the C treatment. The negative effect of monensin on nitro-metabolizing bacteria was supported by the nitroethane-concentration, which showed higher values on 2NEM in comparison with 1NE and 2NE, displayed a low utilization of NE with the addition of monensin. The sensitivity of these bacteria to monensin could be explained in part because monensin has been recognized to negatively affect gram-positive bacteria, specially producers of lactic, acetic, butyric and formic acids and H_2 as main end products (Miller and Wolin, 2001; Tedeschi et al., 2003), and the most important identified nitro-metabolizing bacteria is *Denitrobacterium detoxificans*, a gram-positive nitro-respiring microorganism (Anderson et al., 2000c). Nevertheless the mechanism by which the nitro-metabolizing bacteria was affected is unknown and may be attributed to

the lack of the outer membrane, ion selectivity of the ionophore, or an increased in ion flux through the cell membrane. However, the ability of gram-positive ruminal bacteria to resist monensin has been documented (Callaway et al., 1999).

Previous results have shown adaptation evidence of rumen microbial populations to nitroethane (Gutierrez-Banuelos et al., 2007). The MPN of nitro-metabolizing bacterial populations displayed that nitroethane treatment specifically 1NE and 2NE increased in comparison to the 2NEM and the C. Also previous results indicated that nitroethane treatment produced a relatively stable population of ruminal bacteria capable of 3-nitro-1-propanol and NO_2^- degradation (Majak et al., 1986).

Conclusions

These results confirm the methane-inhibiting activity of nitroethane, and suggest that ruminal adaptation of bacteria to nitroethane is likely due to an enrichment of nitro-metabolizing bacteria. The results further demonstrate that monensin treatment negatively affected nitro-metabolizing bacterial populations.

CHAPTER V

EFFECTS OF TANNINS ON FOODBORNE PATHOGENS AS PREHARVEST INTERVENTIONS

Introduction

Foodborne pathogens including *Campylobacter*, Shiga toxin-producing *Escherichia coli* (STEC O157) and STEC non-O157 are found in the gastrointestinal tract of ruminants. *Campylobacter* has been observed to be low or absent at the rumen level and present with great variability in lower intestine (Gutierrez-Banuelos et al., 2007). *Escherichia coli* may be influenced by feeding and nutritional management (Brownlie and Grau, 1967; Callaway et al., 2003; Grau et al., 1969). Fasting stress and sporadic feeding have been shown to increase ruminal and fecal concentrations of total coliforms, however, these factors have had inconsistent effects on *E. coli* O157:H7 (Rasmussen et al., 1999). Recently, the prevalence for *E. coli* O157:H7 on US feedlot cattle was reported at 13% (LeJeune et al., 2004), and as high as 89% for *Campylobacter* (Stanley and Jones, 2003). According to surveillance by Foodborne Diseases Active Surveillance Network (FoodNet) of the CDC, the overall disease incidence (laboratory confirmed cases) per 100,000 populations was 12.72 for *Campylobacter*, second only to *Salmonella* (14.55). Incidence rates for Shiga toxin-producing *Escherichia coli* O157 (STEC O157) were 1.06, and 0.33 for STEC non-O157 (Vugia et al., 2006). In addition to post-harvest strategies, intervention methods to reduce pathogens loads before arrival

to the slaughter plants may significantly decrease human illness associated with foodborne pathogens (Hynes and Wachsmuth, 2000; Vugia et al., 2003).

Dietary plant tannins are phenolic compounds chemically categorized as condensed (proanthocyanidins) or hydrolyzable (Gallate esters) tannins (Van Soest, 1994), although a combination of both in different proportions have been more common (Kumar and Vaithiyanathan, 1990). Nutritional effects of tannins have been reviewed and include decreasing ruminal digestibility of feed proteins, anthelmintic effects against intestinal nematods, prevention and treatment of bloat, and antimicrobial activity (McMahon et al., 2000; Min et al., 2003; Scalbert, 1991). Phenolic compounds have been shown to decrease *Listeria monocytogenes* (Payne et al., 1989) or serve as antifungal agents (Rosenthal et al., 1997). In vitro studies have shown inhibitory effects of condensed tannins against generic *E. coli* and *E. coli* O157 (Min et al., 2007). The mechanisms by which tannins inhibit bacteria have not been fully characterized, but cranberry tannins have been shown to inhibit the adherence of uropathogenic *E. coli* (Zafiri et al., 1989), which was corroborated with studies in women urinary tract infections decreasing their disease risk with consumption of cranberry juice (Kontiokari et al., 2003). The inhibitory effect of tannic acid on the growth of intestinal bacteria has been proposed to be due to the strong iron capacity (Chung et al., 1998), whereas, the antimicrobial effects of green tea catechins and epigallocatechin have been linked to actions in the outer polar zone of lipid bilayers in liposomes (Hashimoto et al., 1999; Terao et al., 1994).

The objectives of this experiment were to test the effects of two sources of tannins, mimosa- and chestnut-tannin, when applied as an antimicrobial hide-spray intervention against generic *E. coli*, total coliforms and total aerobic bacterial loads and as a feed supplement against generic *E. coli*, total coliforms, and *Campylobacter* spp. in steers fed high grain diets.

Materials and Methods

Animals

Approval for care and use of animals used in this study (AUP 2007-27) was obtained from the Institutional animal care and use committee of Texas A & M University. Thirty-six crossbreed steers averaging 414 kg (± 40 ; SD) were stratified by initial BW and randomly assigned to one of three treatments ($n = 12$): control (CN), mimosa tannin (MT), and chestnut tannin (CT). Commercially available tannin extracts, chestnut tannin (*Castanea sativa* Mill; containing about 80% hydrolyzable tannins) and mimosa tannin (*Acacia mearnsii*, black wattle; containing about 70% condensed tannins) (CHEMTAN® CHESTNUT POWDER KPN and CHEMTAN® MIMOSA ME POWDER, Chemptan Co, NH, USA, 03833) were supplemented in a total mixed ration at 1.5% of the diet. Within treatments, steers were assigned to one of two pens (6 steers/pen), each equipped with Calan-gate feeders and transitioned to high-grain based diet during a 28 d adaptation period. Three step-up rations were fed for one week each and the final finishing diet fed during the last week of the 28 d adaptation period (Table 5.1). The finishing diet was formulated to meet nutritional requirements to support growth rates of 1.5 kg/d (NRC., 1996). The experimental phase, which tested 42 d, was

TABLE 5.1. Ration formulations used on the adaptation and experimental phase on finishing steers treated with tannins

Ingredient	Step-up rations			Treatment diets*		
	Week 1	Week 2	Week 3	Control	Mimosa	Chestnut
Corn	38.5	49.7	63.4	71.5	70.0	70.0
Hay-sorghum	38.0	27.8	18.1	8.0	8.0	8.0
CSH [†]	10.0	7.0	4.0	2.0	2.0	2.0
CSM ^{††}	4.0	6.0	6.0	11.0	11.0	11.0
Molasses	6.0	6.0	5.0	4.0	4.0	4.0
Limestone	1.5	1.5	1.5	1.5	1.5	1.5
TM/Vit [£]	2.0	2.0	2.0	2.0	2.0	2.0
Tannin mix	0.0	0.0	0.0	0.0	1.5	1.5

*The treatment diets were formulated to meet nutritional requirements to support growth rates at 1.5 kg/d

[†]Cottonseed hulls

^{††}Cottonseed meal

[£]Trace minerals and vitamins supplement

completed in two replicates. Each replicate consisted of one pen for each treatment, with the second replicate being initiated one week following the first replicate. To avoid possible cross contamination, the same pens were used to evaluate the hide-spray and feed supplemented treatments.

Hide-swabs Bacteriological Sampling

Starting on d 13 of the adaptation phase, a 1000 cm² hide-surface area was marked on each animal's area left flank, by positioning the top line below the costal vertebra and the left line at the back of the shoulder. The marked hide-surface area was divided into four equal-size quadrants and each quadrant identified by clipping the hair around the borders of quadrants. The quadrants were designated A, B, C and D beginning at the top front quadrant and proceeding clockwise. Previously penned steers were treated with CN (distilled water), MT or CT solutions. The tannin treatment solutions were prepared by mixing the MT and CT extracts in distilled water at 3% (wt/vol). Treatment solutions were sprayed at low-pressure at approximately 15 cm from the surface until the hide quadrants were equally wet (but not running) (25 mL/1000 cm²) with a pump action bottle-type hand sprayer. Hide-swab samples were obtained after three-directional swabbing (12 swabs per quadrant) using Speci-sponges (Nasco, Fort Atkinson, WI), which were hydrated with 25 ml of 2X concentration of Dey-Engley neutralization broth (Difco, Detroit, MI). They were placed in sample bags and transported to the lab for bacteriological cultivations of *E. coli*/total coliforms, and total aerobes. Hide-swabs samples were obtained at pre-spray, 2 min, 8 h, and 24 h post-spray application (corresponding to quadrants A, B, C and D).

Ruminal Fluid and Fecal Bacteriological Sampling

Rumen fluid samples were obtained by stomach tube on d 0, 7, 21 and 42 of the experimental period and placed into 50 mL plastic tubes. Freshly voided feces were obtained weekly prior to feeding (06:30) from d 0 to d 42 and placed in air-tight plastic bags. Collected samples were immediately returned to the lab for bacteriological quantification. Rumen fluid and fecal samples were achieved via plating for *E. coli*/total coliforms. *Campylobacter* was plated only on d 0 and 7.

Bacteriological Cultivation

Hide-swabs sample bags were stomached for one minute before dilution. Fecal samples (1 g), ruminal fluid (1 mL) and hide-swab fluid (1 mL) were serially diluted (10-fold) into 9 mL of 0.1 M phosphate buffer (pH 6.5). Dilutions of hide swab samples were plated to *E. coli*/coliform Petrifilms and APC Petrifilms (3M Microbiology, St. Paul, MN) to enumerate *E. coli*/total coliforms and total aerobes in hide-swabs samples. Dilutions of ruminal and fecal samples were plated similarly to enumerate *E. coli*/total coliforms and were also plated to Campy Cefex agar to enumerate *Campylobacter*. After 24 h incubation at 37 °C incubations of *E. coli*/total coliforms, and aerobic bacteria colonies were visualized and enumerated following manufacturer's instructions. After 48 h of incubation at 42 °C colonies that exhibited typical *Campylobacter* morphology were counted.

Statistical Analysis

Colony forming units (CFU) of *E. coli*, total coliforms, total aerobes, and *Campylobacter* were numerically transformed (\log_{10}) prior to statistical analysis. The

statistical model for ruminal fluid and fecal data included the effects of treatment, day of treatment, and their interaction in a repeated measures analysis of variance. The statistical model for hide-swabs includes the effects for treatment, swab-time, and their interaction in a repeated measures analysis of variance. For all analyses, replicate was included in the models as a random effect. Tukey's protected LSM test ($P < 0.05$) was used to assess treatment differences. For all the analysis PROC MIXED (SAS v 9.1; SAS Inst. Inc., Cary, NC) was used.

Results and Discussions

Effects of Tannin-spray Application on Hide-swab Bacteriological Load

There were no tannin treatment or time of sampling effects ($P > 0.05$) on bacteriological counts of hide-swab samples (Table 5.2), which averaged 1.7, 1.5, and 1.5 for *E. coli*, 1.8, 1.6, and 1.7 for total coliforms, and 4.0, 3.4, and 4.2 for total aerobic count (\log_{10} CFU/cm²) for CN, CT, and MT treatments, respectively. Previous research has indicated the importance of hides as a key pathogen-transmitter to beef carcasses, especially for Shiga toxin-producing *E. coli* O157 and non-O157 (Barkocy-Gallagher et al., 2003; Barkocy-Gallagher et al., 2001). Considering that previous in vitro studies showed an inhibitory effect of condensed tannins against *E. coli* (Min et al., 2007), we decided to evaluate two sources of condensed tannins (mimosa and chestnut) as a potential antimicrobial intervention on beef cattle hides. The methodology used in this study to examine effectiveness of tannin spray application may have been limited due to animal-to-animal cross contamination, possible loss of tannin compound efficacy during dehydration while on the hide, or neutralization of phenolic-mediated bacterial-binding

Table 5.2. Effects of tannin spray application as hide-intervention to decrease foodborne pathogens

	Treatment ^a			SEM	P
	Control	Chestnut	Mimosa		
<i>E. coli</i> ^b				0.3	
Pre-spray	1.8	1.4	1.4		
Post-spray (2 min)	2.1	1.6	1.2		
Post-spray (8 h)	1.5	1.4	1.4		
Post-spray (24 h)	1.4	1.7	1.8		
Treatment effect					0.29
Time effect					0.64
Interaction					0.10
Total coliforms ^b				0.3	
Pre-spray	1.9	1.5	1.6		
Post-spray (2 min)	2.2	1.7	1.5		
Post-spray (8 h)	1.6	1.5	1.5		
Post-spray (24 h)	1.5	1.8	2.1		
Treatment effect					0.46
Time effect					0.31
Interaction					0.12
Aerobic plate count ^b				0.2	
Pre-spray	4.1	4.2	4.4		
Post-spray (2 min)	4.2	4.0	4.0		
Post-spray (8 h)	3.7	3.9	4.0		
Post-spray (24 h)	3.9	3.9	4.4		
Treatment effect					0.17
Time effect					0.09
Interaction					0.36

^aTreatments tested included 3% tannin solutions diluted on distilled water. ^blog₁₀

CFU/cm².

capacity within the Speci-sponge sampling matrix. The Dey-Engley neutralization broth used to rehydrate the Speci-sponges has been shown to neutralize phenols (Dey and Engley, 1995, 1994).

Effects of Tannin Feed Supplementation on Bacteriological Load in Rumen Fluid and Fecal Samples

Previous studies have not examined the effects of condensed tannins (mimosa- and chestnut-tannin) on *E. coli*, total coliforms or *Campylobacter* counts in ruminants fed high-grain diets. Ruminal samples from steers supplemented with CT had 0.5 and 0.4 log (CFU) lower ($P < 0.01$) counts of *E. coli* and total coliforms, respectively in comparison with the CN steers, with ruminal samples for steers supplemented with MT being intermediate. Nevertheless, CT did not display the same antimicrobial effect on feces in this trial. Fecal *E. coli* load was not affected ($P > 0.05$) by CT, but MT showed higher amounts ($P < 0.01$), both in comparison with the CN treatment. Moreover, total coliform loads were higher by 0.3 and 0.4 log, respectively, in fecal samples for CT and MT supplemented steers compared to controls (Table 5.3). However, Min et al. (Min et al., 2007) reported that condensed tannins reduced the specific bacterial growth rate of *E. coli* O157:H7 in vitro when compared with the non-tannin control. In contrast to results of this study, the same authors reported that chestnut tannin decreased fecal *E. coli* shedding on high-forage fed animals (Min et al., 2007). Chestnut tannins which consist primarily of hydrolyzable tannins, have been shown to be degraded in the anaerobic environment of the intestinal tract (Nelson et al., 1995). However, it has not been

Table 5.3. Effects of feed-supplementation of two sources of tannins against selected foodborne pathogens on feedlot steers

	Treatment [#]			SEM	P
	Control	Chestnut	Mimosa		
Ruminal Content*					
<i>E.coli</i>	4.1 ^B	3.6 ^A	3.8 ^{AB}	0.1	
Treatment effect					< 0.01
Day effect					0.13
Interaction					0.52
Total coliforms	4.2 ^B	3.8 ^A	4.0 ^{AB}	0.1	
Treatment effect					< 0.01
Day effect					0.12
Interaction					0.72
Fecal content**					
<i>E.coli</i>	5.6 ^A	5.8 ^{AB}	5.9 ^B	0.1	
Treatment effect					< 0.01
Day effect					0.16
Interaction					0.34
Total coliforms	5.7 ^A	6.0 ^B	6.1 ^B	0.1	
Treatment effect					< 0.001
Day effect					0.06
Interaction					0.29
<i>Campylobacter</i> [†]				0.3	
d0	0.1 ^{ab}	0.05 ^a	0.4 ^{ab}		
d7	0.7 ^{bc}	2.0 ^d	1.2 ^c		
Treatment effect					< 0.05
Day effect					< 0.001
Interaction					< 0.01

^{AB}Means within a row without a common letter differ ($P < 0.05$).

^{abcd}Means without a common letter differ ($P < 0.05$).

[#]Treatments tested included 1.5% as feed base of two sources of condensed tannins.

[†]Because *Campylobacter* was almost absent at the rumen level no statistical analysis was reported.

*Ruminal contents are in log₁₀ CFU/mL.

**Fecal contents are in log₁₀ CFU/g.

reported the probably mechanism of action of chestnut tannin on generic *E. coli* on steers. Scalbert (Scalbert, 1991) suggested that *E. coli* was capable of using tannins as a unique source of carbon, but the source of tannin was not specified. Again, the apparent antimicrobial effect of CT tannin was not maintained beyond the rumen in this experiment. Consumption of grain-based diets have been related to increase slime-producing bacteria, such as *Streptococcus bovis*, which produces a dextran slime that increases the viscosity of ruminal fluid (McMahon et al., 2000). *Streptococcus caprinus* has been observed to tolerate high levels of tannins, producing exopolysaccharide which could bind tannins and produce cell coat polymers (O'Donovan and Brooker, 1995). With that in mind, there is a possibility that physical changes produced by diet, especially in viscosity of rumen fluid that was observed in this experiment (data not showed) and previously reported (Meyer and Bartley, 1971), decreased the possible antimicrobial activity of tannins. Nevertheless, dietary proanthocyanidins have been reported to establish tannin-resistant bacteria in the rat gastrointestinal tract, which according to full-length 16S rRNA gene sequence analysis corresponded to strains of *E. coli* and *Bacteroides thetaiotaomicron* (Smith and Mackie, 2004). The resistance mechanism under anaerobic conditions has yet to be elucidated; however, an increase in the oxidative stress response could assist *E. coli* strains to defeat the inhibitory effect of condensed tannins under aerobic conditions (Smith et al., 2003).

It has been observed that the prevalence and load of *Campylobacter* in the rumen is low or almost absent (Gutierrez-Banuelos et al., 2007), which agrees with the results in this experiment as *Campylobacter* was recovered from the rumen of only 3 and 23

steers and average 1.3 ± 0.3 and 1.9 ± 0.7 log CFU/ml on d 0 and d 7 respectively in those animals from which *Campylobacter* was recovered. Consequently, because of the low load or absence of *Campylobacter* in the ruminal samples, a statistical analysis of treatment effects were not performed. Treatment, day of sampling, and treatment by day effects ($P < 0.05$) were observed for *Campylobacter* counts in fecal samples in this experiment (Table 5.3). No differences were observed ($P > 0.05$) on day 0 among treatments, however, *Campylobacter* counts were increased on day 7 with the addition of CT and MT by < 0.8 log when compared with day 0. In contrast, no differences were observed on *Campylobacter* counts between days on CN. Previously reported data showed that phlorotannins, tannins derived from brown algae (*Ecklonia kurome*), had bactericidal activity against *Campylobacter* spp. in vitro, actually, this bacteria was the most susceptible of that study which included strains of *E. coli*, *S. aureus*, *Bacillus cereus*, among others (Nagayama et al., 2002). Tannins enhance the availability of amino acids to the lower gut via protection of proteins from rumen degradation (Min et al., 2003). Thus, the increase in *Campylobacter* concentrations in feces of tannin-treated steers is not unexpected as *Campylobacter*, being asaccharolytic, depends largely on amino acid fermentation for conservation of energy. Also, apparent transmission of *Campylobacter jejuni* was observed between feedlot cattle during the feeding period, resulting in a high prevalence of *C. jejuni* excretion by cattle approaching slaughter (Besser et al., 2005).

Because the importance of *E. coli* and *Campylobacter* as foodborne pathogens, as well as the necessity of natural alternative products to decrease foodborne pathogens,

more research is necessary to understand the action mode of condensed tannins, especially chestnut tannin, which showed an antibacterial activity at the rumen. Also, the lack of knowledge about the diet effects, high-forage or high-grain diets, and the effects of tannins in various gastrointestinal regions open interesting research fields.

Conclusions

In conclusions, the results demonstrated that under our research conditions neither mimosa nor chestnut tannins were effective in reducing hide bacterial load counts of *E. coli*, total coliforms, or total aerobic count. Thus, these tannin sources would not be effective for use in pre-harvest strategies to decrease hide foodborne pathogens load. Nevertheless, more research is necessary under a controlled environment and using different concentrations to test the possible antimicrobial effect of chestnut and mimosa tannins. Chestnut treatment decreased *E. coli* load in rumen fluid, but fail to decrease the load in feces. Because an apparent inactivation of the antimicrobial chestnut tannin effect was present after the rumen level, more research is necessary to elucidate the possible effects of the diet and its changes on physical and chemical characteristics at the different gastrointestinal levels.

CHAPTER VI

GENERAL CONCLUSIONS

The continuous demand of beef (meat) is arising parallel with beef production; also, the risk of foodborne pathogens, especially Shiga toxin-producing *Escherichia coli* (STEC O157), STEC non-O157 and *Campylobacter*. These bacteria are frequently found at the gastrointestinal tract and on hides. Alternative strategies to decrease foodborne pathogens have a better chance to be implemented if they have an economic impact for producers. In this dissertation, alternative strategies that could have a negative impact on bacteriological pathogen load and also a probably anti-methanogenic effect were tested. Because ruminal CH₄ emissions from cattle are representing an important wasteful process correlating its inhibition with an improvement on feed efficiency and also CH₄ is recognized as a potent greenhouse gas involved on global warming, strategies that could affect both, foodborne pathogens and methane production, they have a better opportunity to be implemented being economically feasible.

Results from the first study showed that oral chlorate administration effectively decrease fecal *E. coli* in feedlot cattle. However, this was not enhanced by prior nitroethane treatment. Also, nitroethane did not support our hypothesis that this nitrocompound negatively affects *Campylobacter*. The lack of bactericidal activity could be attributed to rapid absorption, expiration and rumen microbial degradation. However, nitroethane proved be a stronger antimethanogenic compound reducing methane-specific activity although these results were not corroborated with the SF₆ technique of whole

animal emissions. Also this experiment provided evidence of a nitroethane dose-dependent antimethanogenic effect as well as an adaptation of the rumen to lower doses of nitroethane (Chapter III). Using the MPN, we provided evidence (Chapter IV) that adaptation of the rumen microbes to low-dose nitroethane could be due to an increase in nitro-metabolizing bacterial populations, those populations also showed being sensible to monensin. In both experiments (Chapters III and IV), VFA were not greatly affected by nitroethane.

Under our research conditions two sources of tannins, chestnut and mimosa, they were not effective as pre-harvest strategies to decrease foodborne pathogens on hides. However, it is recommended more investigation under a control environment and with other dose levels (Chapter V). Chestnut tannins decrease load of *E. coli* and total coliforms but fail to decrease those foodborne pathogens on feces. More research is recommended to evaluate the possible bactericidal effect of chestnut tannin.

Because the experimental chlorate, nitroethane, and the chestnut and mimosa tannins are not approved for the United States Department of Agriculture, further studies will be necessary to more fully elucidate the mode of action, and to better understand the limits of their activity, as well as physiological implications for the animal. Also, those compounds have to be tested to discard toxic effects on the whole animal or accumulation of dangerous residues in organs and tissues.

LITERATURE CITED

- Allison, C., and G. T. Macfarlane. 1988. Effect of nitrate on methane production and fermentation by slurries of human fecal bacteria. *J. Gen. Microbiol.* 134: 1397-1405.
- Allison, M. J., W. R. Mayberry, C. S. McSweeney, and D. A. Stahl. 1992. *Synergistes jonesii*, gen. Nov., sp. Nov.: A ruminal bacterium that degrades toxic pyridinediols. *Syst. Appl. Microbiol.* 15: 522-529.
- Anderson, R. C., S. A. Buckley, T. R. Callaway, K. J. Genovese, L. F. Kubena, R. B. Harvey, and D. J. Nisbet. 2001. Effect of sodium chlorate on *Salmonella* Typhimurium concentrations in the weaned pig gut. *J. Food Prot.* 64: 255-258.
- Anderson, R. C., S. A. Buckley, L. F. Kubena, L. H. Stanker, R. B. Harvey, and D. J. Nisbet. 2000a. Bactericidal effect of sodium chlorate on *Escherichia coli* O157:H7 and *Salmonella* Typhimurium dt104 in rumen contents in vitro. *J. Food Prot.* 63: 1038-1042.
- Anderson, R. C., T. R. Callaway, T. J. Anderson, L. F. Kubena, N. K. Keith, and D. J. Nisbet. 2002. Bactericidal effect of sodium chlorate on *Escherichia coli* concentrations in bovine ruminal and fecal concentrations in vivo. *Microb. Ecol. Health Dis.* 14: 24-29.
- Anderson, R. C., T. R. Callaway, J. A. Van Kessel, Y. S. Jung, T. S. Edrington, and D. J. Nisbet. 2003. Effect of select nitrocompounds on ruminal fermentation; an initial

look at their potential to reduce economic and environmental costs associated with ruminal methanogenesis. *Bioresour. Technol.* 90: 59-63.

Anderson, R. C., M. A. Carr, R. K. Miller, D. A. King, G. E. Carstens, K. J. Genovese, T. R. Callaway, T. S. Edrington, Y. Soo Jung, J. L. McReynolds, M. E. Hume, R. C. Beier, R. O. Elder, and D. J. Nisbet. 2005a. Effects of experimental chlorate preparations as feed and water supplements on *Escherichia coli* colonization and contamination of beef cattle and carcasses. *Food Microb.* 22: 439-447.

Anderson, R. C., G. E. Carstens, R. K. Miller, T. R. Callaway, C. L. Schultz, T. S. Edrington, R. B. Harvey, and D. J. Nisbet. 2006a. Effect of oral nitroethane and 2-nitropropanol administration on methane-producing activity and volatile fatty acid production in the ovine rumen. *Bioresour. Technol.* 97: 2421-2426.

Anderson, R. C., K. J. Genovese, R. B. Harvey, L. H. Stanker, J. R. DeLoach, and D. J. Nisbet. 2000b. Assessment of the long-term shedding pattern of *Salmonella* serovar choleraesuis following experimental infection of neonatal piglets. *J. Vet. Diagn. Invest.* 12: 257-260.

Anderson, R. C., R. B. Harvey, J. A. Byrd, T. R. Callaway, K. J. Genovese, T. S. Edrington, Y. S. Jung, J. L. McReynolds, and D. J. Nisbet. 2005b. Novel preharvest strategies involving the use of experimental chlorate preparations and nitro-based compounds to prevent colonization of food-producing animals by foodborne pathogens. *Poultry Sci.* 84: 649-654.

Anderson, R. C., M. E. Hume, K. J. Genovese, T. R. Callaway, Y. S. Jung, T. S. Edrington, T. L. Poole, R. B. Harvey, K. M. Bischoff, and D. J. Nisbet. 2004.

Effect of drinking-water administration of experimental chlorate ion preparations on *Salmonella enterica* serovar Typhimurium colonization in weaned and finished pigs. Vet. Res. Commun. 28: 179-189.

Anderson, R. C., Y. S. Jung, K. J. Genovese, J. L. McReynolds, T. R. Callaway, T. S. Edrington, R. B. Harvey, and D. J. Nisbet. 2006b. Low level nitrate or nitroethane preconditioning enhances the bactericidal effect of suboptimal experimental chlorate treatment against *Escherichia coli* and *Salmonella* typhimurium but not *Campylobacter* in swine. Foodborne Pathog. Dis. 3: 461-465.

Anderson, R. C., Y. S. Jung, C. E. Oliver, S. M. Horrocks, K. J. Genovese, R. B. Harvey, T. R. Callaway, T. S. Edrington, and D. J. Nisbet. 2007. Effects of nitrate or nitro supplementation, with or without added chlorate, on *Salmonella enterica* serovar Typhimurium and *Escherichia coli* in swine feces. J. Food Prot. 70: 308-315.

Anderson, R. C., W. Majak, M. A. Rasmussen, T. R. Callaway, R. C. Beier, D. J. Nisbet, and M. J. Allison. 2005c. Toxicity and metabolism of the conjugates of 3-nitropropanol and 3-nitropropionic acid in forages poisonous to livestock. J. Agric. Food Chem. 53: 2344-2350.

Anderson, R. C., and M. A. Rasmussen. 1998. Use of a novel nitrotoxin-metabolizing bacterium to reduce ruminal methane production. Bioresour. Technol. 64: 89-95.

- Anderson, R. C., M. A. Rasmussen, and M. J. Allison. 1993. Metabolism of the plant toxins nitropropionic acid and nitropropanol by ruminal microorganisms. *Appl. Environ. Microbiol.* 59: 3056-3061.
- Anderson, R. C., M. A. Rasmussen, and M. J. Allison. 1996. Enrichment and isolation of a nitropropanol-metabolizing bacterium from the rumen. *Appl. Environ. Microbiol.* 62: 3885-3886.
- Anderson, R. C., M. A. Rasmussen, A. A. DiSpirito, and M. J. Allison. 1997. Characteristics of a nitropropanol-metabolizing bacterium isolated from the rumen. *Can. J. Microbiol.* 43: 617-624.
- Anderson, R. C., M. A. Rasmussen, N. S. Jensen, and M. J. Allison. 2000c. *Denitrobacterium detoxificans* gen. Nov., sp. Nov., a ruminal bacterium that respire on nitrocompounds. *Int. J. Syst. Evol. Microbiol.* 50: 633-638.
- Anderson, R. C., Rasmussen, M. A. 1998. Use of a novel nitrotoxin-metabolizing bacterium to reduce ruminal methane production. *Bioresour. Technol.* 64: 89-95.
- AOAC. 1980. Official Methods of Analysis. 13th ed. Assoc. Off. Anal. Chem., Arlington, Va.
- Bae, W., D. D. Hancock, D. R. Call, Y. H. Park, A. C. B. Berge, R. M. Finger, W. M. Sischo, and T. E. Besser. 2007. Dissemination of antimicrobial resistant strains of *Campylobacter coli* and *Campylobacter jejuni* among cattle in Washington state and California. *Vet. Microbiol.* 122: 306-315.

- Bae, W., K. N. Kaya, D. D. Hancock, D. R. Call, Y. H. Park, and T. E. Besser. 2005. Prevalence and antimicrobial resistance of thermophilic *Campylobacter* spp. from cattle farms in Washington state. *Appl. Environ. Microbiol.* 71: 169-174.
- Barkocy-Gallagher, G. A., T. M. Arthur, M. Rivera-Betancourt, X. Nou, S. D. Shackelford, T. L. Wheeler, and M. Koohmaraie. 2003. Seasonal prevalence of shiga toxin-producing *Escherichia coli*, including O157:H7 and non-O157 serotypes, and *Salmonella* in commercial beef processing plants. *J. Food Prot.* 66: 1978-1986.
- Barkocy-Gallagher, G. A., T. M. Arthur, G. R. Siragusa, J. E. Keen, R. O. Elder, W. W. Laegreid, and M. Koohmaraie. 2001. Genotypic analyses of *Escherichia coli* O157:H7 and O157 nonmotile isolates recovered from beef cattle and carcasses at processing plants in the Midwestern states of the United States. *Appl. Environ. Microbiol.* 67: 3810-3818.
- Beach, J. C., E. A. Murano, and G. R. Acuff. 2002. Prevalence of *Salmonella* and *Campylobacter* in beef cattle from transport to slaughter. *J. Food Prot.* 65: 1687-1693.
- Besser, T. E., J. T. LeJeune, D. H. Rice, J. Berg, R. P. Stilborn, K. Kaya, W. Bae, and D. D. Hancock. 2005. Increasing prevalence of *Campylobacter jejuni* in feedlot cattle through the feeding period. *Appl. Environ. Microbiol.* 71: 5752-5758.
- Božić, A., R. C. Anderson, G. E. Carstens, S. C. Ricke, T. R. Callaway, and M. T. Yokoyama. 2005. Effects of nitroethane, lauric acid, lauricidin® and the hawaiian marine algae, *Chaetoceros*, on ruminal methane production and some

zoonotic pathogens in vitro. Pages 440-443 in 2nd International Conference of Greenhouse Gases and Animal Agriculture GGAA, Zurich, Sitzerland.

Brashears, M. M., M. L. Galyean, G. H. Loneragan, J. E. Mann, and K. Killinger-Mann.

2003a. Prevalence of *Escherichia coli* O157:H7 and performance by beef feedlot cattle given *Lactobacillus* direct-fed microbials. J. Food Prot. 66: 748-754.

Brashears, M. M., D. Jaroni, and J. Trimble. 2003b. Isolation, selection, and

characterization of lactic acid bacteria for a competitive exclusion product to reduce shedding of *Escherichia coli* O157:H7 in cattle. J. Food Prot. 66: 355-363.

Brown, E. G., G. E. Carstens, L. J. Slay, S. A. Woods, M. J. Quinn, and J. L.

McReynolds. 2005. Effects of nitroethane administration on methane production in growing steers. J. Anim. Sci. suppl 1: 34.

Brownlie, L. E., and F. H. Grau. 1967. Effect of food intake on growth and survival of

Salmonellas and *Escherichia* in the bovine rumen. J. Gen. Microbiol. 46: 125-134.

Bryant, M. P., and L. A. Burkey. 1953. Cultural methods and some characteristics of

some of the more numerous groups of bacteria in the bovine rumen. J. Dairy Sci. 36: 205-217.

Callaway, T. R., K. A. Adams, and J. B. Russell. 1999. The ability of "Low g + c gram-

positive" ruminal bacteria to resist monensin and counteract potassium depletion. Current Microb. 39: 226-230.

- Callaway, T. R., R. C. Anderson, K. J. Genovese, T. L. Poole, T. J. Anderson, J. A. Byrd, L. F. Kubena, and D. J. Nisbet. 2002. Sodium chlorate supplementation reduces *E. coli* O157:H7 populations in cattle. *J. Anim. Sci.* 80: 1683-1689.
- Callaway, T. R., A. M. S. Carneiro de Melo, and J. B. Russell. 1997. The effect of nisin and monensin on ruminal fermentations in vitro. *Current Microb.* 35: 90-96.
- Callaway, T. R., R. O. Elder, J. E. Keen, R. C. Anderson, and D. J. Nisbet. 2003. Forage feeding to reduce preharvest *Escherichia coli* populations in cattle, a review. *J. Dairy Sci.* 86: 852-860.
- Callaway, T. R., and S. A. Martin. 1996. Effects of organic acid and monensin treatment on in vitro mixed ruminal microorganism fermentation of cracked corn. *J. Anim. Sci.* 74: 1982-1989.
- CDC. 2005. *Campylobacter* infections. Division of bacterial and mycotic diseases. Available: http://www.cdc.gov/ncidod/dbmd/diseaseinfo/campylobacter_g.htm Accessed Oct. 15, 2007
- CDC. 2006. *Escherichia coli* O157:H7. Division of bacterial and mycotic diseases. Available: http://www.cdc.gov/ncidod/dbmd/diseaseinfo/escherichiacoli_g.htm Accessed Oct. 17, 2007
- Chagan, I., and U. Kazunari. 2004. Detection of methanogens and proteobacteria from a single cell of rumen ciliate protozoa. *J. Gen. Appl. Microbiol.* 50: 203-212.
- Chaney, A. L., and E. P. Marbach. 1962. Modified reagents for determination of urea and ammonia. *Clin. Chem.* 8: 130.

- Chhour, K.-L., M. A. Nadkarni, R. Byun, F. E. Martin, N. A. Jacques, and N. Hunter. 2005. Molecular analysis of microbial diversity in advanced caries. *J. Clin. Microbiol.* 43: 843-849.
- Chung, K. T., Z. Lu, and M. W. Chou. 1998. Mechanism of inhibition of tannic acid and related compounds on the growth of intestinal bacteria. *Food Chem. Toxicol.* 36: 1053-1060.
- Deppenmeier, U. 2002. The unique biochemistry of methanogenesis. *Prog. Nucleic Acid Res. Mol. Biol.* 71: 223-283.
- de Vaux, A., M. Morrison, and R. W. Hutkins. 2002. Displacement of *Escherichia coli* O157 : H7 from rumen medium containing prebiotic sugars. *Appl. Environ. Microbiol.* 68: 519-524.
- Dey, B. P., and J. F. B. Engley. 1994. Neutralization of antimicrobial chemicals by recovery media. *J. Microbiol. Methods* 19: 51-58.
- Dey, B. P., and J. F. B. Engley. 1995. Comparison of Dey and Engley (d/e) neutralizing medium to letheen medium and standard methods medium for recovery of *Staphylococcus aureus* from sanitized surfaces. *J. Ind. Microbiol.* 14: 21-25.
- Diez-Gonzalez, F., T. R. Callaway, M. G. Kizoulis, and J. B. Russell. 1998. Grain feeding and the dissemination of acid-resistant *Escherichia coli* from cattle. *Science* 281: 1666-1668.
- Dimitrijevic, M., R. C. Anderson, T. R. Callaway, Y. S. Jung, R. B. Harvey, S. C. Ricke, and D. J. Nisbet. 2006. Inhibitory effect of select nitrocompounds on growth and survivability of *Listeria monocytogenes* in vitro. *J. Food Prot.* 69: 1061-1065.

- Doyle, M. P., and M. C. Erickson. 2006. Reducing the carriage of foodborne pathogens in livestock and poultry. *Poult. Sci.* 85: 960-973.
- Edrington, T. S., T. R. Callaway, R. C. Anderson, K. J. Genovese, Y. S. Jung, J. L. McReynolds, K. M. Bischoff, and D. J. Nisbet. 2003. Reduction of *E. coli* O157:H7 populations in sheep by supplementation of an experimental sodium chlorate product. *Small Ruminant Res.* 49: 173-181.
- Elder, R. O., J. E. Keen, T. Edrington, T. R. Callaway, R. Anderson, and D. Nisbet. 2003. Intervention to reduce fecal shedding of enterohemorrhagic *Escherichia coli* O157:H7 in fed beef cattle. Page 94 in 5th International Symposium on Shiga toxin (Verocytotoxin)- producing *Escherichia coli* infections, Edinburgh, Scotland.
- Elder, R. O., J. E. Keen, G. R. Siragusa, G. A. Barkocy-Gallagher, M. Koohmaraie, and W. W. Laegreid. 2000. Correlation of enterohemorrhagic *Escherichia coli* O157 prevalence in feces, hides, and carcasses of beef cattle during processing. *PNAS* 97: 2999-3003.
- Fox, J. T., R. C. Anderson, G. E. Carstens, R. K. Miller, Y. S. Jung, and J. L. McReynolds. 2005. Effect of nitrate adaption on the bactericidal activity of an experimental chlorate product against *Escherichia coli* in cattle. *Int. J. Appl. Res. Vet. Med.* 3: 76-80.
- Garcia, J. L., B. K. C. Patel, and B. Ollivier. 2000. Taxonomic phylogenetic and ecological diversity of methanogenic archaea. *Anaerobe* 6: 205-226.

- Garcia, M. M., H. Lior, R. B. Stewart, G. M. Ruckerbauer, J. R. R. Trudel, and A. Skljarevski. 1985. Isolation, characterization, and serotyping of *Campylobacter jejuni* and *Campylobacter coli* from slaughter cattle. *Appl. Environ. Microbiol.* 49: 667-672.
- Garcia-Lopez, P. M., L. Kung, Jr., and J. M. Odom. 1996. In vitro inhibition of microbial methane production by 9,10-anthraquinone. *J. Anim. Sci.* 74: 2276-2284.
- Gibson, G. R., and M. B. Roberfroid. 1995. Dietary modulation of the human colonic microbiota - introducing the concept of prebiotics. *J. Nutr.* 125: 1401-1412.
- Grau, F. H., L. E. Brownlie, and M. G. Smith. 1969. Effects of food intake on numbers of *Salmonellae* and *Escherichia coli* in rumen and feces of sheep. *J. Appl. Bacteriol.* 32: 112-117.
- Gutierrez-Banuelos, H., R. C. Anderson, G. E. Carstens, L. J. Slay, N. Ramlachan, S. M. Horrocks, T. R. Callaway, T. S. Edrington, and D. J. Nisbet. 2007. Zoonotic bacterial populations, gut fermentation characteristics and methane production in feedlot steers during oral nitroethane treatment and after the feeding of an experimental chlorate product. *Anaerobe* 13: 21-31.
- Harper, R. J., A. C. Beck, P. Ritson, M. J. Hill, C. D. Mitchell, D. J. Barrett, K. R. J. Smettem, and S. S. Mann. 2007. The potential of greenhouse sinks to underwrite improved land management. *Ecol. Engin.* 29: 329-341.

- Hashimoto, T., S. Kumazawa, F. Nanjo, Y. Hara, and T. Nakayama. 1999. Interaction of tea catechins with lipid bilayers investigated with liposome systems. *Biosci. Biotechnol. Biochem.* 63: 2252-2255.
- Hinton, A., Jr., D. E. Corrier, G. E. Spates, J. O. Norman, R. L. Ziprin, R. C. Beier, and J. R. DeLoach. 1990. Biological control of *Salmonella* Typhimurium in young chickens. *Avian Dis.* 34: 626-633.
- Hong, J., J. M. Kim, W. K. Jung, S. H. Kim, W. Bae, H. C. Koo, J. Gil, M. Kim, J. Ser, and Y. H. Park. 2007. Prevalence and antibiotic resistance of *Campylobacter* spp. isolated from chicken meat, pork, and beef in Korea, from 2001 to 2006. *J. Food Prot.* 70: 860-866.
- Horrocks, S. M., Y. S. Jung, J. K. Huwe, R. B. Harvey, S. C. Ricke, G. E. Carstens, T. R. Callaway, R. C. Anderson, N. Ramlachan, and D. J. Nisbet. 2007. Effects of short-chain nitrocompounds against *Campylobacter jejuni* and *Campylobacter coli* in vitro. *J. Food Sci.* 72: M50-M55.
- Horrocks, S. M., Y. S. Jung, S. C. Ricke, T. R. Callaway, T. S. Edrington, and R. B. Harvey. 2005. Effects of nitroethane and 2-nitropropanol against *Campylobacter jejuni*. Pages 194-196 in *Proceedings 6th International Symposium on the Epidemiology and Control of Foodborne Pathogens in Pork*, Rohnert Park, CA, USA.
- Howell, A. B., and B. Foxman. 2002. Cranberry juice and adhesion of antibiotic-resistant uropathogens. *JAMA.* 287: 3082-3083.

- Hristov, A. N., M. Ivan, L. Neill, and T. A. McAllister. 2003. Evaluation of several potential bioactive agents for reducing protozoal activity in vitro. *Anim. Feed Sci. Technol.* 105: 163-184.
- Hynes, N. A., and I. K. Wachsmuth. 2000. *Escherichia coli* O157:H7 risk assessment in ground beef: A public health tool. Page 46 in 4th Int. Symp. and Workshop on Shiga Toxin (Verocytotoxin)-Producing *Escherichia coli* Infections, Kyoto, Japan.
- Johnson, K. A., M. T. Huyler, H. H. Westberg, B. R. Lamb, and P. Zimmerman. 1994. Measurement of methane emissions from ruminant livestock using SF6 tracer technique. *Env. Sci. Tech.* 28: 359-362.
- Johnson, K. A., and D. E. Johnson. 1995. Methane emissions from cattle. *J. Anim Sci.* 73: 2483-2492.
- Jung, Y. S., R. C. Anderson, T. R. Callaway, T. S. Edrington, K. J. Genovese, R. B. Harvey, T. L. Poole, and D. J. Nisbet. 2004a. Inhibitory activity of 2-nitropropanol against select food-borne pathogens in vitro. *Lett. Appl. Microbiol.* 39: 471-476.
- Jung, Y. S., R. C. Anderson, T. S. Edrington, K. J. Genovese, J. A. Byrd, T. R. Callaway, and D. J. Nisbet. 2004b. Experimental use of 2-nitropropanol for reduction of *Salmonella typhimurium* in the ceca of broiler chicks. *J. Food Prot.* 67: 1945-1947.
- Jung, Y. S., R. C. Anderson, K. J. Genovese, T. S. Edrington, T. R. Callaway, and J. Byrd. 2003. Reduction of *Campylobacter* and *Salmonella* in pigs treated with

- selected nitrocompound. Pages 205-207 in Proceedings of the 5th International Symposium on the Epidemiology and Control of Foodborne Pathogens in Pork, Hersonissos, Crete.
- Kim, W. K., R. C. Anderson, A. L. Ratliff, D. J. Nisbet, and S. C. Ricke. 2006. Growth inhibition by nitrocompounds of selected uric acid-utilizing microorganisms isolated from poultry manure. *J. Environ. Sci.* 41: 97-107.
- Kontiokari, T., J. Laitinen, L. Jarvi, T. Pokka, K. Sundqvist, and M. Uhari. 2003. Dietary factors protecting women from urinary tract infection. *Am. J. Clin. Nutr.* 77: 600-604.
- Kumar, R., and S. Vaithyanathan. 1990. Occurrence, nutritional significance and effect on animal productivity of tannins in tree leaves. *Anim. Feed Sci. Technol.* 30: 21-38.
- LeJeune, J. T., T. E. Besser, D. H. Rice, J. L. Berg, R. P. Stilborn, and D. D. Hancock. 2004. Longitudinal study of fecal shedding of *Escherichia coli* O157:H7 in feedlot cattle: Predominance and persistence of specific clonal types despite massive cattle population turnover. *Appl. Environ. Microbiol.* 70: 377-384.
- LeJeune, J. T., and A. N. Wetzel. 2007. Preharvest control of *Escherichia coli* O157 in cattle. *J. Anim. Sci.* 85: E73-E80.
- Leyendecker, S. A., T. R. Callaway, R. C. Anderson, and D. J. Nisbet. 2004. Technical note on a much simplified method for collecting ruminal fluid using a nylon paint strainer. *J. Sci. Food Agric.* 84: 387-389.

- Lin, C. Z., L. Raskin, and D. A. Stahl. 1997. Microbial community structure in gastrointestinal tracts of domestic animals: Comparative analyses using rRNA-targeted oligonucleotide probes. *FEMS Microbiol. Ecol.* 22: 281-294.
- Mack, D. R., S. Michail, S. Wei, L. McDougall, and M. A. Hollingsworth. 1999. Probiotics inhibit enteropathogenic *E. coli* adherence in vitro by inducing intestinal mucin gene expression. *Amer. J. of Physiology-Gastrointestinal and Liver Phys.* 276: G941-G950.
- Majak, W. 1992. Further enhancement of nitropropanol detoxification by ruminal bacteria in cattle. *Can. J. Anim. Sci.* 72: 863-870.
- Majak, W., and K. J. Cheng. 1981. Identification of rumen bacteria that anaerobically degrade aliphatic nitrotoxins. *Can. J. Microbiol.* 27: 646-650.
- Majak, W., K. J. Cheng, and J. W. Hall. 1986. Enhanced degradation of 3-nitropropanol by ruminal microorganisms. *J. Anim. Sci.* 62: 1072-1080.
- Martin, S. A., M. N. Streeter, D. J. Nisbet, G. M. Hill, and S. E. Williams. 1999. Effects of dl-malate on ruminal metabolism and performance of cattle fed a high-concentrate diet. *J. Anim. Sci.* 77: 1008-1015.
- McGinn, S. M., K. A. Beauchemin, T. Coates, and D. Colombatto. 2004. Methane emissions from beef cattle: Effects of monensin, sunflower oil, enzymes, yeast, and fumaric acid. *J. Anim. Sci.* 82: 3346-3356.
- McMahon, L. R., T. A. McAllister, B. P. Berg, W. Majak, S. N. Acharya, J. D. Popp, B. E. Coulman, Y. Wang, and K. J. Cheng. 2000. A review of the effects of forage

condensed tannins on ruminal fermentation and bloat in grazing cattle. Can. J. Plant. Sci. 80: 469-485.

McReynolds, J., L. Kubena, J. Byrd, R. Anderson, S. Ricke, and D. Nisbet. 2005.

Evaluation of *Salmonella enteritidis* in molting hens after administration of an experimental chlorate product (for nine days) in the drinking water and feeding an alfalfa molt diet. Poult. Sci. 84: 1186-1190.

McReynolds, J. L., J. A. Byrd, R. W. Moore, R. C. Anderson, T. L. Poole, T. S.

Edrington, L. F. Kubena, and D. J. Nisbet. 2004. Utilization of the nitrate reductase enzymatic pathway to reduce enteric pathogens in chickens. Poult. Sci. 83: 1857-1860.

Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, and C. Shapiro. 1999.

Food-related illness and death in the United States. Emerg. Inf. Dis. 5: 607-625.

Meyer, R. M., and E. E. Bartley. 1971. Bloat in cattle. Xv. The relation of viscosity and cell-free polysaccharide content of rumen fluid to feedlot bloat No. 33. p 1018-1021.

Miller, T. L. 1995. Ecology of methane production and hydrogen sinks in the rumen.

Pages 317-331 in Ruminant Physiology: Digestion, metabolism, growth and reproduction, Stuttgart, Germany.

Miller, T. L., and M. J. Wolin. 2001. Inhibition of growth of methane-producing bacteria of the ruminant forestomach by hydroxymethylglutaryl-scoa reductase inhibitors. J. Dairy Sci. 84: 1445-1448.

- Min, B. R., T. N. Barry, G. T. Attwood, and W. C. McNabb. 2003. The effect of condensed tannins on the nutrition and health of ruminants fed fresh temperate forages: A review. *Anim. Feed Sci. and Techn.* 106: 3-19.
- Min, B. R., W. E. Pinchak, R. C. Anderson, and T. R. Callaway. 2007. Effect of tannins on the in vitro growth of *Escherichia coli* O157:H7 and in vivo growth of generic *Escherichia coli* excreted from steers. *J. of Food Protec.* 70: 543-550.
- Min, B. R., W. E. Pinchak, R. C. Anderson, J. D. Fulford, and R. Puchala. 2006. Effects of condensed tannins supplementation level on weight gain and in vitro and in vivo bloat precursors in steers grazing winter wheat. *J. Anim Sci.* 84: 2546-2554.
- Min, B. R., W. E. Pinchak, J. D. Fulford, and R. Puchala. 2005. Wheat pasture bloat dynamics, in vitro ruminal gas production, and potential bloat mitigation with condensed tannins. *J. Anim Sci.* 83: 1322-1331.
- Mohammed, N., Z. A. Lila, N. Ajisaka, K. Hara, K. Mikuni, K. Hara, S. Kanda, and H. Itabashi. 2004. Inhibition of ruminal microbial methane production by beta-cyclodextrin iodopropane, malate and their combination in vitro. *J. Anim. Physiol. Anim. Nutr.* 88: 188-195.
- Moreno-Vivian, C., P. Cabello, M. Martinez-Luque, R. Blasco, and F. Castillo. 1999. Prokaryotic nitrate reduction: Molecular properties and functional distinction among bacterial nitrate reductases. *J Bacteriol.* 181: 6573-6584.
- Moss, R. A., J.-P. Jouany, and J. Newbold. 2000. Methane production by ruminants: Its contribution to global warming. *Ann. Zootech.* 49: 231-253.

- Nagaraja, T. G., C. J. Newbold, C. J. Van Nevel, and D. I. Demeyer. 1997. Manipulation of ruminal fermentation. Pages 523-632 in *The Rumen Microbial Ecosystem*. P. N. Hobson and C. S. Stewart. 2nd ed. Blackie Academic and Profesional. London.
- Nagayama, K., Y. Iwamura, T. Shibata, I. Hirayama, and T. Nakamura. 2002. Bactericidal activity of phlorotannins from the brown alga *Ecklonia kurome*. *J. Antimicrob. Chemother.* 50: 889-893.
- Nelson, K. E., A. N. Pell, P. Schofield, and S. Zinder. 1995. Isolation and characterization of an anaerobic ruminal bacterium capable of degrading hydrolyzable tannins. *Appl. Environ. Microbiol.* 61: 3293-3298.
- Newbold, C. J., Rode, L.M. 2005. Dietary additives to control methanogenesis in the rumen. Pages 60-70 in *Greenhouse Gases and Animal Agriculture GGAA*, ETH Zurich.
- Nocek, J. E., and J. B. Russell. 1988. Protein and energy as an integrated system - relationship of ruminal protein and carbohydrate availability to microbial synthesis and milk-production. *J. Dairy Sci.* 71: 2070-2107.
- Nohynek, L. J., H.-L. Alakomi, M. P. Kahkonen, M. Heinonen, I. M. Helander, K.-M. Oksman-Caldentey, and R. H. Puupponen-Pimia. 2006. Berry phenolics: Antimicrobial properties and mechanisms of action against severe human pathogens. *Nutr. Cancer* 54: 18-32.
- NRC. 1996. *Nutrient Requirements of Beef Cattle*. 7th rev. ed. Natl. Acad. Press, Washington, DC.

- O'Donovan, L. A., and J. D. Brooker. 1995. Exopolysaccharide production by *Streptococcus caprinus* - defense against tannins?. Pages 1-2 in Proceedings of Austriac Society of Biochemistry and Molecular Biology, Austria.
- Okine, E. K., G. W. Mathison, and R. T. Hardin. 1989. Effects of changes in frequency of reticular contractions on fluid and particulate passage rates in cattle. 67: 3388-3396.
- Payne, K. D., E. Rico-Munzo, and P. M. Davidson. 1989. Antimicrobial activity of phenolic compounds against *Listeria monocytogenes* and their effectiveness in a model system. J. Food Prot. 52: 151-153.
- Paynter, M. J., and R. E. Hungate. 1968. Characterization of *Methanobacterium mobilis*, sp. N., isolated from the bovine rumen. J. Bacteriol. 95: 1943-1951.
- Pol, A., and D. I. Demeyer. 1988. Fermentation of methanol in the sheep rumen. Appl. Environ. Microbiol. 54: 832-834.
- Potter, A. A., S. Klashinsky, Y. Li, E. Frey, H. Townsend, D. Rogan, G. Erickson, S. Hinkley, T. Klopfenstein, R. A. Moxley, D. R. Smith, and B. B. Finlay. 2004. Decreased shedding of *Escherichia coli* O157:H7 by cattle following vaccination with type iii secreted proteins. Vaccine 22: 362-369.
- Rasmussen, M. A., T. L. Wickman, J. W. C. Cray, and T. A. Casey. 1999. *Escherichia coli* O157:H7 and the rumen environment. Pages 39-50 in *Escherichia coli* O157 in Farm Animals. C. S. Stewart and H. J. Flint. 1st ed. CAB International Publishing. Wallingford, UK.

- Rios, J. L., and P. G. Waterman. 1997. A review of the pharmacology and toxicology of astragalus. *Phytotherapy. Res.* 11:411-418.
- Rosenthal, I., B. Rosen, and S. Bernstein. 1997. Phenols in milk evaluation of ferulic acid and other phenols as antifungal agents. *Milchwissenschaft* 52: 134-138.
- Russell, J. B., and A. J. Houlihan. 2003. Ionophore resistance of ruminal bacteria and its potential impact on human health. *FEMS Microbiol. Rev.* 27: 65-74.
- Russell, J. B., and H. J. Strobel. 1989. Effect of ionophores on ruminal fermentation. *Appl. Environ. Microbiol.* 55: 1-6.
- Russell, J. B., and R. J. Wallace. 1988. Energy yielding and consuming reactions. Pages 185-216 in *The Rumen Microbial Ecosystem*. Elsevier Applied Science. London.
- Saengkerdsab, S., W. K. Kim, R. C. Anderson, D. J. Nisbet, and S. C. Ricke. 2006. Effects of nitro compounds and feedstuffs on in vitro methane production in chicken cecal contents and rumen fluid. *Anaerobe* 12: 85-92.
- Santoso, B., B. Mwenya, C. Sar, Y. Gamo, T. Kobayashi, R. Morikawa, K. Kimura, H. Mizukoshi, and J. Takahashi. 2004. Effects of supplementing galacto-oligosaccharides, *Yucca schidigera* or nisin on rumen methanogenesis, nitrogen and energy metabolism in sheep. *Livest. Prod. Sci.* 91: 209-217.
- Scalbert, A. 1991. Antimicrobial properties of tannins. *Phytochem.* 30: 3875-3883.
- Schamberger, G. P., R. L. Phillips, J. L. Jacobs, and F. Diez-Gonzalez. 2004. Reduction of *Escherichia coli* O157:H7 populations in cattle by addition of colicin e7-producing *E. coli* to feed. *Appl. Environ. Microbiol.* 70: 6053-6060.

- Smith, A. H., J. A. Imlay, and R. I. Mackie. 2003. Increasing the oxidative stress response allows *Escherichia coli* to overcome inhibitory effects of condensed tannins. *Appl. Environ. Microbiol.* 69: 3406-3411.
- Smith, A. H., and R. I. Mackie. 2004. Effect of condensed tannins on bacterial diversity and metabolic activity in the rat gastrointestinal tract. *Appl. Environ. Microbiol.* 70: 1104-1115.
- Smith, K. E., J. M. Besser, C. W. Hedberg, F. T. Leano, J. B. Bender, J. H. Wicklund, B. P. Johnson, K. A. Moore, M. T. Osterholm, I. Team. 1999. Quinolone-resistant *Campylobacter jejuni* infections in Minnesota, 1992-1998. *N. Engl. J. Med.* 340: 1525-1532.
- Stanley, K., and K. Jones. 2003. Cattle and sheep farms as reservoirs of *Campylobacter*. *J. Appl. Microbiol.* 94: 104S-113S.
- Stern, N. J., B. Wojton, and K. A. Kwiatier. 1992. A differential selective medium, and dry-ice generated atmosphere for recovery of *Campylobacter jejuni*. *J. Food Prot.* 55: 514-517.
- Stewart, C. S., and M. P. Bryant. 1988. The rumen bacteria. Pages 21-76 in *The Rumen Microbial Ecosystem*. P. N. Hobson. Elsevier Applied Science. London.
- Stewart, V. 1988. Nitrate respiration in relation to facultative metabolism in enterobacteria. *Microbiol. Rev.* 52: 190-232.
- Tauxe, R. V. 2002. Emerging foodborne pathogens. *Int. J. Food Microbiol.* 78: 31-41.
- Tedeschi, L. O., D. G. Fox, and T. P. Tylutki. 2003. Potential environmental benefits of ionophores in ruminant diets. *J. Environ. Qual.* 32: 1591-1602.

- Terao, J., M. Piskula, and Q. Yao. 1994. Protective effect of epicatechin, epicatechin gallate, and quercetin on lipid-peroxidation in phospholipid-bilayers. *Arch. Biochem. Biophys.* 308: 278-284.
- Theodorou, M. K., D. J. Gascoyne, D. E. Akin, and R. D. Hartley. 1987. Effect of phenolic acids and phenolics from plant cell walls on rumenlike fermentation in consecutive batch culture. *Appl. Environ. Microbiol.* 53: 1046-1050.
- Thornton, J. H., and F. N. Owens. 1981. Monensin supplementation and in vivo methane production by steers. *J. Anim. Sci.* 52: 628-634.
- Tokura, M., K. Ushida, K. Miyazaki, and Y. Kojima. 1997. Methanogens associated with rumen ciliates. *FEMS Microbiol. Ecol.* 22: 137-143.
- UN. 1998. Kyoto protocol to the United Nations framework convention on climate change, UN, New York.
- Ungerfeld, E. M., S. R. Rust, and R. Burnett. 2003. Use of some novel alternative electron sinks to inhibit ruminal methanogenesis. *Reprod. Nutr. Dev.* 43: 189-202.
- Ungerfeld, E. M., S. R. Rust, and R. Burnett. 2006. Effects of butyrate precursors on electron relocation when methanogenesis is inhibited in ruminal mixed cultures. *Lett. Appl. Microbiol.* 42: 567-572.
- USDA/ERS. 2004. U.S. Department of Agriculture. Economic Research Service. ERS estimates foodborne disease costs at \$6.9 billion per year. Available at: <http://www.ers.usda.gov/briefing/FoodborneDisease/features.htm>.

- Van Nevel, C. J., and D. I. Demeyer. 1995. Feed additives and other interventions for decreasing methane emissions. Pages 329-349 in *Biotechnology in Animal Feeds and Animal Feeding*. R. J. Wallace and A. Chesson. Wiley-Verlag. Weinheim, Germany.
- Van Nevel, C. J., and D. I. Demeyer. 1996. Control of rumen methanogenesis. *Environ. Monit. Assess.* 42: 73-79.
- Van Soest, P. J. 1994. Plant defensive chemicals. Pages 196-212 in *Nutritional Ecology of the Ruminant*. P. J. Van Soest. Cornell University Press, Ithaca, NY.
- Vugia, D., A. Cronquist, J. Hadler, M. Tobin-D'angelo, D. Blythe, K. Smith, K. Thornton, D. Morse, P. Cieslak, T. Jones, K. Holt, J. Guzewich, O. Henao, E. Scallan, F. Angulo, P. Griffin, R. Tauxe, and E. Barzilay. 2006. Preliminary foodnet data on the incidence of infection with pathogens transmitted commonly through food, 10 states, United States, 2005. *MMWR* 55: 392-395.
- Vugia, D., J. Hadler, S. Chaves, D. Blythe, K. Smith, and D. Morse. 2003. Preliminary foodnet data on the incidence of foodborne illness-selected sites, United States. *MMWR* 52: 340-343.
- Wallace R. J., L. A., and C.J. Newbold. 1994. Influence of *Yucca shidigera* extract on ruminal ammonia concentrations and ruminal microorganisms. *Appl. Environ. Microbiol.* 60: 1762-1767.
- Wallace, R. J., R. Onodera, and M. A. Cotta. 1997. Metabolism of nitrogen-containing compounds. Pages 283-328 in *The Rumen Microbial Ecosystem*. P. N. Hobson and C. S. Stewart. 2nd ed. Blackie Academic and Professional. London.

- Wang, Y., T. A. McAllister, C. J. Newbold, L. M. Rode, P. R. Cheeke, and K. J. Cheng. 1998. Effects of *Yucca shidigera* extract on fermentation and degradation of steroidal saponins in the rumen simulation technique (rusitec). Anim. Feed Sci. Technol. 74: 143-153.
- White, D. G., S. Zhao, R. Singh, and P. F. McDermott. 2004. Antimicrobial resistance among gram-negative foodborne bacterial pathogens associated with foods of animal origin. Foodborne Pathog. Dis. 1: 137-152.
- Williams, Y. J., S. M. Rea, S. Popovski, C. L. Pimm, A. J. Williams, A. F. Toovey, L. C. Skillman, and A. D. Wright. 2007. Responses of sheep to a vaccination of entodinal or mixed rumen protozoal antigens to reduce rumen protozoal numbers. Br. J. Nutr. 1-10.
- Wolin, M. J. 1960. A theoretical rumen fermentation balance. J. Dairy Sci. 43: 1452-1459.
- Wright, A. D. G., C. H. Auckland, and D. H. Lynn. 2007. Molecular diversity of methanogens in feedlot cattle from Ontario and Prince Edward Island, Canada. Appl. Environ. Microbiol. 73: 4206-4210.
- Wright, A. D., P. Kennedy, C. J. O'Neill, A. F. Toovey, S. Popovski, S. M. Rea, C. L. Pimm, and L. Klein. 2004a. Reducing methane emissions in sheep by immunization against rumen methanogens. Vaccine 22: 3976-3985.
- Wright, A. D. G., A. J. Williams, B. Winder, C. T. Christophersen, S. L. Rodgers, and K. D. Smith. 2004b. Molecular diversity of rumen methanogens from sheep in Western Australia. Appl. Environ. Microbiol. 70: 1263-1270.

- Zafriri, D., I. Ofek, M. Pocino, and N. Sharon. 1989. Inhibitory activity of cranberry juice on adherence of type 1 and type p fimbriated *Escherichia coli* to eucaryotic cells. *Antim. Agents and Chemot.* 33: 92-98.
- Zhao, T., M. P. Doyle, B. G. Harmon, C. A. Brown, P. O. E. Mueller, and A. H. Parks. 1998. Reduction of carriage of enterohemorrhagic *Escherichia coli* O157:H7 in cattle by inoculation with probiotic bacteria. *J. Clin. Microbiol.* 36: 641-647.
- Zhao, T., S. Tkalcic, M. P. Doyle, B. G. Harmon, C. A. Brown, and P. Zhao. 2003. Pathogenicity of enterohemorrhagic *Escherichia coli* in neonatal calves and evaluation of fecal shedding by treatment with probiotic *Escherichia coli*. *J. Food Prot.* 66: 924-930.

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